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Determination of phytochemical content, antioxidant and mineral properties of *Astragalus chamaephaca* Freyn in different solvents

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Astragalus species, members of the Fabaceae family, have been used in traditional medicine for centuries. In addition to their traditional applications, many subspecies are now utilized in modern medicine. This study investigated the antioxidative, phenolic, and mineralogical properties of *Astragalus chamaephaca* Freyn for the first time. Antioxidant capacities were evaluated using the ABTS⁺ free cation radical scavenging activity, FRAP, and DPPH methods. The total phenolic content was determined using the Folin-Ciocalteu method, while quantification was performed via the LC-MS method. Substantial phenolic compounds, such as *p*-coumaric acid, gallic acid, rutin, quercetin, and naringenin—known for their diverse biological activities—were identified. In addition, mineralogical analyses have shown the presence of essential elements, such as Ca, Mg, and K, which play crucial roles in human health.

Keywords *Astragalus*, Phenolic content, Antioxidant activity, *p*-coumaric acid, Mineralogical properties

Free radicals are constantly formed in the human body through natural processes. When their levels rise above a certain threshold, they can damage healthy cells due to their unstable chemical structure, increasing the risk of diseases such as cancer, diabetes, Alzheimer's, and heart disease. This condition, which is characterized by elevated free radical levels, is known as oxidative stress. Antioxidants are substances that cation and neutralize free radicals, thereby reducing oxidative stress¹. Phenolic and flavonoids are increasingly recognized as major bioactive components that contribute to the antioxidant potential of many plants². These antioxidants can be derived from plants, animals, and microorganisms. Medicinal and aromatic plants have historically played a crucial role in treating a wide range of diseases. Phenolic compounds are the main contributors to the therapeutic properties of plants. Numerous studies have highlighted the protective role of plant phenolics against cancer, inflammation, and degenerative diseases, including cardiovascular problems, diabetes, and more³. Plants are fascinating for their ability to produce raw materials or preparations containing phytochemicals with significant antioxidant capacities and health benefits⁴. The World Health Organization's 2014–2023 strategy seeks to promote the use of traditional medicines, including herbal medicines, to keep people healthy by providing access to adequate and affordable alternatives to medicines and offering health choices that align with people's cultural practices⁵.

Astragalus L. is the largest genus in the family Fabaceae (subfamily Papilionideae, tribe Galegeae). It is widely distributed worldwide, with many species found across continents such as Asia, Europe, North America, and Africa. *Astragalus* spp. has been used in traditional medicine for centuries⁶. There are many studies on extracts obtained from the parts of these commercially valuable plants and their antioxidant properties, such as *Astragalus cicer* and *A. glycyphyllos*⁷, *A. tribuloides*⁸, *A. membranaceus*⁹, *A. flavesens*¹⁰, *A. armatus*¹¹, *A. sinicus*¹². The studies, which examined different parts of the plant, reported that *Astragalus* L. and its subspecies are essential sources of antioxidants and beneficial against many diseases. Tan et al. found that the extracts obtained from *A. membranaceus* are an effective source of antioxidants for the food and medical industries¹³. Muhammed et al. Reported *Astragalus* spp. provides substantial defense and protection against damage to the heart, brain, kidneys,

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intestines, liver, and lungs in various disease models caused by oxidative stress¹⁴. Li et al. speculated that, based on standard criteria for antitumor activity, *Astragalus*L. polysaccharides exhibit moderate to high antitumor activity in tumor cells¹⁵. Wei et al., in their in vitro tests, stated that polysaccharides obtained from *Astragalus*L. have anti-inflammatory effects¹⁶.

Astragalus chamaephaca Freyn was first described in Turkey in 1911 by the Hungarian botanist Andrasovszky¹⁷. Türkiye is home to 481 taxa belonging to 64 sections, 44.9% of which are endemic to the country^{18–21}. Although studies have identified varying distributions of these taxa in Anatolia, their antioxidant activities and mineralogical analyses have not been carried out until now^{22–27}.

In this study, 2,2-diphenyl-1-picrylhydrazil (DPPH) radical scavenging activity, ABTS⁺, Ferric Ions (Fe3+) Reducing Antioxidant Power (FRAP), Total Phenolic Content, and mineralogical analysis were carried out to investigate the antioxidant activity of *A. chamaephaca*'s leaf and flower extracts obtained from different solvents. Phenolic compounds were identified and quantified using UHPLC-HESI-MS/MS.

Materials and methods

Reagents and plant materials

Potassium ferricyanide [K₃Fe(CN)₆], DPPH, Trichloroacetic acid (TCA), FeCl₃, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin-Ciocalteu reagent, Na₂SO₄ (anhydrous sodium sulfate), Na₂CO₃ (sodium carbonate), ABTS⁺, Potassium persulfate, phosphate buffer, nitric acid and phenolic compounds were obtained from Sigma (Sigma-Aldrich Chemie GmbH, Sternheim, Germany) and used without any purification. Ethanol, methanol, dichloromethane, and Hydrogen peroxide (H₂O₂) were purchased from Merck. In May 2023, *A. chamaephaca* was collected in the region of Çorum (40.660, 34.797) with the approval of the Republic of Türkiye Ministry of Agriculture and Forestry. The collected herbs were identified by Ömer Koray Yaylaci, PhD., who is affiliated with Anadolu University, Medicinal Plants, Drugs, and Scientific Research Centre.

Extraction

A. chamaephaca collected from the Çorum-Laçın /TÜRKİYE region was divided into two parts: flowers and leaves. The samples were dried in the shade and pulverized using a grinder. A total of 500 mg of each part was weighed and placed into tubes. Each sample was combined with 25 mL of one of three solvents, water, methanol, or ethanol, and mixed. The mixtures were kept in an ultrasonic bath at a fixed temperature of 25 °C and a frequency of 53 kHz for 60 min. The mixture was then vortexed thoroughly. All extracts were prepared using the same method and subsequently analyzed for the quantification of phenolic compounds and antioxidant capacities²⁷.

Phenolic compounds identification and quantitation

This study conducted the LC-MS/MS phenolic compound analysis using a Thermo Scientific Dionex Ultimate 3000 UHPLC system coupled with a TSQ Quantum Access Max tandem mass spectrometer. The liquid chromatography system comprised binary pumps, a degasser, a column compartment, and an autosampler. Chromatographic separation was achieved using a C18 reversed-phase Inertsil ODS HYPERSIL analytical column (250 mm × 4.6 mm, 5 µm), with the column temperature maintained at 30 °C. Mobile phase A (water with 0.1% formic acid) and mobile phase B (methanol) were used for the elution gradient. The gradient program was set as follows 0–1 min, 0% B; 1–22 min, 95% B; 22–25 min, 95% B; 25–30 min, 100% B. The total evaluation time, including the conditioning period, was 34 min. The injection volume and solvent flow rate were adjusted to 20 µL and 0.7 mL/min, respectively, to achieve the desired outcome. After a series of trials to optimize ionization and molecule separation conditions, the aforementioned mobile phase was selected for use in this study. The conditions for the analysis were determined based on the study by Kayir et al.²⁷. Limit of Detection (LOD) and Limit of Quantification (LOQ) values were calculated by using the signal-to-noise (S/N) method. The calculations were performed according to the following equations; LOD = 3*S/N, LOQ = 10*S/N²⁸. The compounds listed in Table 1 were analyzed using LC-MS/MS and chromatograms of the phenolic compound standards are given as supplementary files (S1–S5). Total Ion Chromatogram (TIC) is a chromatogram created by summing up intensities of all mass spectral peaks belonging to the same scan. Chromatograms containing peaks of 29 phenolic compounds used as standards are shown separately in the total ion chromatogram (TIC) between S1 and S5 in the supplementary material. Since the phenolic standards were prepared as a mixture, there is a single total ion chromatogram (TIC) and this TIC is located on the standard chromatogram figures. For the calibration graph, phenolic compound standard mixtures at six different concentrations were prepared and analyzed in LC-MS/MS. The calibration graph was obtained using the peak area versus concentration of standards. In addition, the total ion chromatograms (TIC) of leaf and flower extracts extracted in different solvents are shown between S6 and S11 in the supplementary material. In the total ion chromatograms (TIC) given between S6 and S11, it was observed that the intensities of the ion peaks changed in proportion to the concentrations of phenolic compounds detected in the plant extracts. The amounts of phenolic compounds detected in the plant extracts were calculated quantitatively with the obtained calibration graphs.

Total phenolic content determination

The total phenolic content of *A. chamaephaca* extracts was determined using the method described by Kayir et al.²⁷, which is a modified version of the Singleton and Slinkard method²⁹. The total phenolic content was calculated using the equation of the standard curve, which was prepared with the standard compound gallic acid equation (GAE/kg dried plant).

Analyte	Parent ion (m/z)	MS/MS (CE)	Ionization Mode	Rt	Regression equation	R ²	RSD%	Standard calibration graph range (mg/L)	LOD (mg/L)	LOQ (mg/L)
Pyrogallol	124.9	69.3 (20)–79.3 (23)	Neg	8.00	$Y = 868,238 \times X$	0.9967	3.5	0.125–4.0	0.058	0.193
Gallic Acid	169.7	80.5 (25)–126.2 (16)	Neg	9.85	$Y = 633,735 \times X + 388,801$	0.9970	4.5	0.125–4.0	0.061	0.203
Protocatechuic acid	155	65.4 (22)–93.2 (13)	Pos	11.77	$Y = 887,836 \times X + 132,867$	0.9984	4.9	0.125–4.0	0.049	0.162
Protocatechuic aldehyde	136.9	92.3 (25)–108.2 (25)	Neg	12.64	$Y = 6.76504e + 006 \times X + 3.68207e + 006$	0.9983	2.2	0.125–4.0	0.026	0.087
Sesamol	137.2	108.3 (15)–109.3 (14)	Neg	12.73	$Y = 3.32578e + 006 \times X + 1.8097e + 006$	0.9953	2.1	0.125–4.0	0.031	0.103
Catechin	289.2	203.9 (22)–245.7 (17)	Neg	12.95	$Y = 5.22198e + 007 \times X + 9.73073e + 006$	0.9977	3.1	0.125–4.0	0.010	0.032
Gentisic acid	153.7	109.5 (21)	Neg	12.99	$Y = 2.27957e + 006 \times X$	0.9962	2.3	0.125–4.0	0.013	0.043
Epicatechin	291.5	123.3 (15)–139.3 (16)	Neg	14.38	$Y = 1.23711e + 008 \times X + 2.28107e + 007$	0.9975	2.5	0.125–4.0	0.003	0.006
Caffeic acid	179.7	135.2 (27)–136.2 (18)	Neg	14.84	$Y = 1.04816e + 007 \times X + 2.40453e + 006$	0.9954	2.5	0.125–4.0	0.042	0.058
Vanillin	150.9	92.3 (23)–136.1 (16)	Neg	15.36	$Y = 4.59206e + 006 \times X - 42736.1$	0.9965	3.7	0.125–4.0	0.023	0.076
Syringic acid	183.07	77.3 (23)–123.2 (13)	Neg	15.76	$Y = 2.79667e + 008 \times X + 7.07664e + 007$	0.9950	4.6	0.125–4.0	0.194	0.647
Syringaldehyde	180.88	151.1 (24)–166.1 (16)	Neg	15.81	$Y = 1.41741e + 007 \times X$	0.9994	4.1	0.125–4.0	0.177	0.590
Taxifolin	303.0	126.2 (23)–285.5 (15)	Neg	16.27	$Y = 5.93177e + 006 \times X + 981,386$	0.9996	3.8	0.125–4.0	0.001	0.005
P-Coumaric acid	163.9	94.3 (33)–120.2 (17)	Neg	16.41	$Y = 1.22044e + 006 \times X + 318,722$	0.9976	3.5	0.125–4.0	0.069	0.109
Ferulic acid	195.0	89.4 (30)–177.4 (7)	Pos	16.77	$Y = 1.33833e + 006 \times X + 369,770$	0.9960	4.5	0.125–4.0	0.063	0.118
Sinapic acid	223.0	149.1 (22)–164.1 (17)	Neg	16.95	$Y = 99251.8 \times X$	0.9988	4.1	0.125–4.0	0.211	0.703
Salicylic acid	137.1	65.5 (35)–93.3 (18)	Neg	17.19	$Y = 4.97191e + 007 \times X + 4.51112e + 006$	0.9998	4.8	0.125–4.0	0.030	0.099
p-hydroxybenzoic acid	137.9	66.6 (38)–94.6 (17)	Neg	17.28	$Y = 4.75093e + 007 \times X + 3.64957e + 006$	0.9997	4.7	0.125–4.0	0.243	0.809
Rosmarinic acid	359.2	134.3 (44)–162.2 (20)	Neg	17.41	$Y = 1.97151e + 007 \times X - 714,706$	0.9996	2.6	0.125–4.0	0.003	0.005
Oleuropein	539.1	275.8 (22)–377.5 (16)	Neg	17.59	$Y = 2.21141e + 007 \times X + 1.87751e + 006$	0.9984	2.0	0.125–4.0	0.009	0.029
Rutin	609.37	300.6 (38)–301.7 (34)	Neg	18.03	$Y = 1.53144e + 008 \times X - 1.12027e + 007$	0.9984	1.6	0.125–4.0	0.022	0.073
Resveratrol	228.9	107.2 (22)–135.1 (14)	Pos	18.14	$Y = 3.62808e + 007 \times X - 149,003$	0.9976	4.8	0.125–4.0	0.019	0.062
Ellagic Acid	301.7	284.8 (30)–174.2 (34)	Neg	19.16	$Y = 1.53671e + 007 \times X - 735,404$	0.9959	5.0	0.125–4.0	0.087	0.289
Cinnamic acid	147.8	78.4 (22)–104.7 (13)	Neg	19.44	$Y = 2.916e + 006 \times X$	0.9991	4.1	0.125–4.0	0.104	0.347
Naringenin	273.0	147.1 (20)–153.0 (24)	Pos	19.71	$Y = 3.82862e + 008 \times X$	0.9949	4.8	0.125–4.0	0.005	0.017
Quercetin	301.0	152.1 (23)–179.9 (20)	Neg	20.38	$Y = 6.92068e + 007 \times X - 587,242$	0.9987	2.9	0.125–4.0	0.001	0.005
Kaempferol	286.9	153.0 (33)–165.0 (28)	Pos	21.46	$Y = 9.52799e + 007 \times X - 6.43708e + 006$	0.9981	4.4	0.125–4.0	0.188	0.447
Galangin	268.9	169.1 (29)–171.1 (31)	Neg	23.43	$Y = 2.00724e + 007 \times X$	0.9979	5.0	0.125–4.0	0.034	0.113
Flavone	222.9	77.3 (35)–121.2 (26)	Pos	23.74	$Y = 1.06423e + 009 \times X - 1.40121e + 008$	0.9976	1.7	0.125–4.0	0.037	0.124

Table 1. Calibration curve, LOD and LOQ information for phenolic compounds in the chromatographic method. Parent ion (m/z): Molecular ions of the standard compounds. MS/MS(CE): SRM fragments for the related molecular ions (CE refers to related collision energies of the fragment ions)

Method of free radical scavenging activity (DPPH.)

The DPPH free radical scavenging activity of *A. chamaephaca* extracts was determined using the Blois method³⁰. Briefly, 1 mL of 0.26 mM DPPH solution was added to 40 μ L of extract solution, and the final volume was adjusted to 4 mL with ethyl alcohol. The mixture was then shaken at room temperature and left to stand for 30 min. The same DPPH solution was used for all solvent extracts. The absorbance was measured on a spectrophotometer at 517 nm. Calculations were performed based on the study by Kayir et al. The results have been calculated as the IC_{50} .

Method of ferric ions (Fe^{3+}) reducing antioxidant power (FRAP)

The FRAP antioxidant power of *A. chamaephaca* extracts was measured by the pristine method³¹. Potassium ferricyanide and phosphate buffer were added to the *A. chamaephaca* extract solutions. TCA and $FeCl_3$ were added after the incubation of the mixture for 20 min at 50 °C. The mixture was then vortexed vigorously. The absorbance of the resulting solution was recorded at 700 nm using a spectrophotometer, as described in the Oyaizu method. A higher absorbance of the reaction mixture indicated a stronger reducing power. The reduction power results were calculated from the equation of the standard curve, which was prepared using the standard compound Trolox (μ mol TE g⁻¹).

Method of free cation radical scavenging activity (ABTS⁺)

The ABTS⁺ (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) activity of *A. chamaephaca* extracts was determined using the Re method³². Briefly, 40 μ L of ABTS⁺- $K_2S_2O_8$ solution (1 mL) was added to the extract solution. Phosphate buffer was then added until the volume of the solution reached 4 mL. The mixture was vortexed at room temperature and left to stand for 30 min. The absorbance was measured on a spectrophotometer at 734 nm. A decrease in absorbance indicated a higher ability to scavenge free cation radicals. Calculations were performed according to the study by Kayir et al.²⁷. The results were calculated as IC_{50} .

Mineral analysis

Samples for ICP-OES mineral analysis were prepared using a Berghof Instruments Speedwave system (Germany). The preparation involved weighing 3 g of sample into Teflon digestion vessels, followed by the careful addition of 2 mL of 30% (w/v) H_2O_2 solution and 5 mL of 65% (w/v) HNO_3 solution using a clean glass pipette. The mixture was thoroughly mixed and allowed to stand for 10 min before the vessels were sealed. The samples then underwent a three-step microwave digestion process: 170 °C for 5 min, 190 °C for 15 min, and 50 °C for 10 min. After cooling, the resulting colorless solutions were quantitatively transferred into 10 mL volumetric flasks and diluted to volume with deionized water³³.

Standard solutions for Ca, Cu, Fe, Na, K, Mg, Mn, P and Zn were prepared by diluting a multi-element ICP QC standard solution (100 mg L⁻¹) obtained from Chem-Lab. (Zedelgem, Belgium). The total concentrations of the elements were determined using inductively coupled plasma optical emission spectrometry (ICP-OES) with a Thermo Scientific iCap 6000 Dual view instrument.

Statistical analysis

The experimental data were analyzed using SPSS Statistics Version 22.0. We utilized the ANOVA test to identify any statistical differences in the results ($p < 0.05$). The results are presented as the mean \pm standard deviation ($n = 3$).

Results and discussion

Total phenolic content

The Folin-Ciocalteu method was employed to determine the total phenolic content of the *A. chamaephaca*. The results are expressed as gallic acid equivalent (GAE). Among the extracts, the methanol extract of the leaf part exhibited the highest total phenolic content, while the methanol extract of the floral part showed the lowest. For the aqueous extracts, the total phenolic content was 713 mg GAE/kg of dried plant for the floral part and 971 mg GAE/kg of dried plant for the leaf part. The values were 632 mg GAE/kg dried plant and 961 mg GAE/kg dried plant, respectively, in ethanolic extracts. The values were 580 mg GAE/kg dried plant and 1109 mg GAE/kg dried plant in the methanol extracts. Phenolic compounds were found to be the lowest in the flower part of the plant in all extracts (Fig. 1).

DPPH. Free radical scavenging activity

The result of the DPPH radical scavenging test to determine the IC_{50} value of two parts of *A. chamaephaca* water, methanol and ethanol extracts are shown in Figure 2. Antiradical effectiveness refers to the quantity required to reduce DPPH levels by 50%. A high IC_{50} value, calculated based on DPPH antioxidant activity, indicates

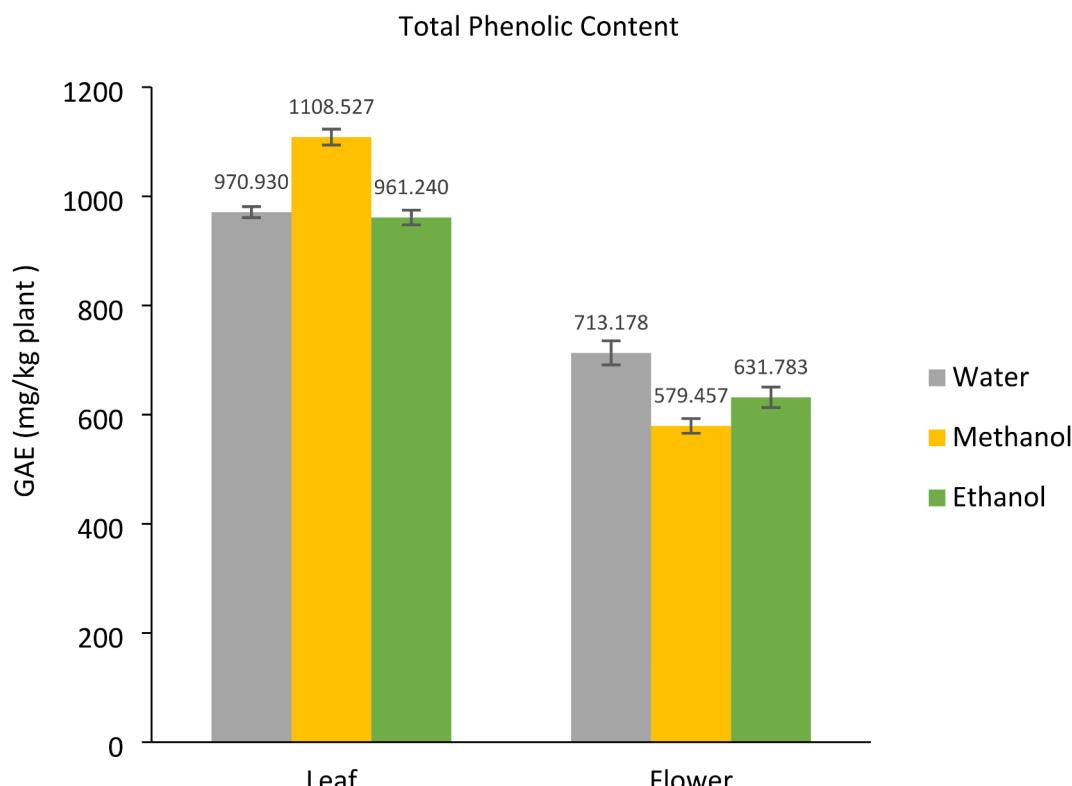


Fig. 1. Total phenolic content of *A. chamaephaca* parts in water, methanol and ethanol extracts by Folin-Ciocalteu method as gallic acid equivalent (GAE).

poor antioxidant activity. According to Fig. 2, the water extract of the plant's leaf showed the highest DPPH antioxidant activity, while ethanol extracts showed the lowest.

IC_{50} values (mg/mL) for different extract solutions of *A. chamaephaca* parts, obtained using the DPPH assay, are presented in Fig. 2. The water extract of the leaf part showed an IC_{50} of 0.007 mg/mL, while the flower part exhibited an IC_{50} of 0.42 mg/mL. When the DPPH antioxidant capacities of the leaf part of the plant in methanol and ethanol extracts were compared with water extracts, it was found that the water extract was approximately 33 times more effective. Additionally, the water extract of *A. chamaephaca* leaves demonstrated a higher antioxidant capacity than BHT (0.021 mg/mL), based on their IC_{50} values.

ABTS⁺ free cation radical scavenging activity

Figure 3 presents the IC_{50} values obtained from ABTS⁺ free cation radical scavenging assays for water, methanol, and ethanol extracts of two parts of *A. chamaephaca*. Anti-radical activity refers to the amount of antioxidants required to reduce the ABTS⁺ concentration by 50%. A high IC_{50} level, calculated based on ABTS⁺ antioxidant activity, signifies lower antioxidant activity. Among the extracts, the water extract of the leaf part exhibited the highest ABTS⁺ antioxidant activity, whereas the ethanol extract showed the lowest antioxidant activity, as shown in Fig. 3.

Ferric ions (Fe³⁺) reducing antioxidant power (FRAP)

The antioxidant activities of the leaf and flower parts of the *A. chamaephaca* extracts, expressed as μ M trolox equivalents (TEAC value) using the FRAP method, are shown in Fig. 4. The methanol extract of the leaf part of the plant exhibited the highest antioxidant capacity. Antioxidant capacity values of 93, 207, and 241 μ M Trolox equivalents were recorded for the flower part in ethanol, methanol and water extracts, respectively. Among all extracts, the ethanolic extract of the flower part showed the lowest FRAP activity.

In literature, studies on different *Astragalus* species have reported varying results. Albayrak et al. conducted a study to determine the total phenolic content in methanol extracts of four different *Astragalus*: *A. gummifer* (8.47 mg GAE/g extract), *A. microcephalus* (10.84 mg GAE/g extract), *A. thalassus* (9.78 mg GAE/g extract), and *A. acmophyllus* (16.38 mg GAE/g extract). They found that each species exhibited a different total phenolic content. The DPPH activity efficiency of *Astragalus* species was determined as follows: *A. gummifer* (IC_{50} = 195.19 μ g/mL), *A. microcephalus* (IC_{50} = 86.67 μ g/mL), *A. thalassus* (IC_{50} = 211.66 μ g/mL), and *A. acmophyllus* (IC_{50} = 253.88 μ g/mL). In addition, the antioxidant capacities of these species were determined in FRAP studies: *A. gummifer* (0.76 mM/L), *A. microcephalus* (0.97 mM/L), *A. thalassus* (0.66 mM/L), and *A. acmophyllus* (0.60 mM/L)³².

Jaradat et al. determined the total phenolic content in methanol extracts from *A. aleppicus*, *A. angustifolius*, *A. annularis*, and *A. boeticus*, reporting values of 35.14 mg GAE/g, 60.14 mg GAE/g, 75.34 mg GAE/g, and 35.54 mg GAE/g, respectively. They concluded that *A. boeticus* has a moderate phenolic content. In their DPPH studies with extracts obtained from water, acetone, methanol, and DCM from *Astragalus* species, they found that methanol extracts exhibited the best antioxidant activity. Specifically, they determined that *A. boeticus* had an $IC_{50} \pm SD$ value of 3.91 ± 0.34 μ g/mL for radical scavenging activity³⁴.

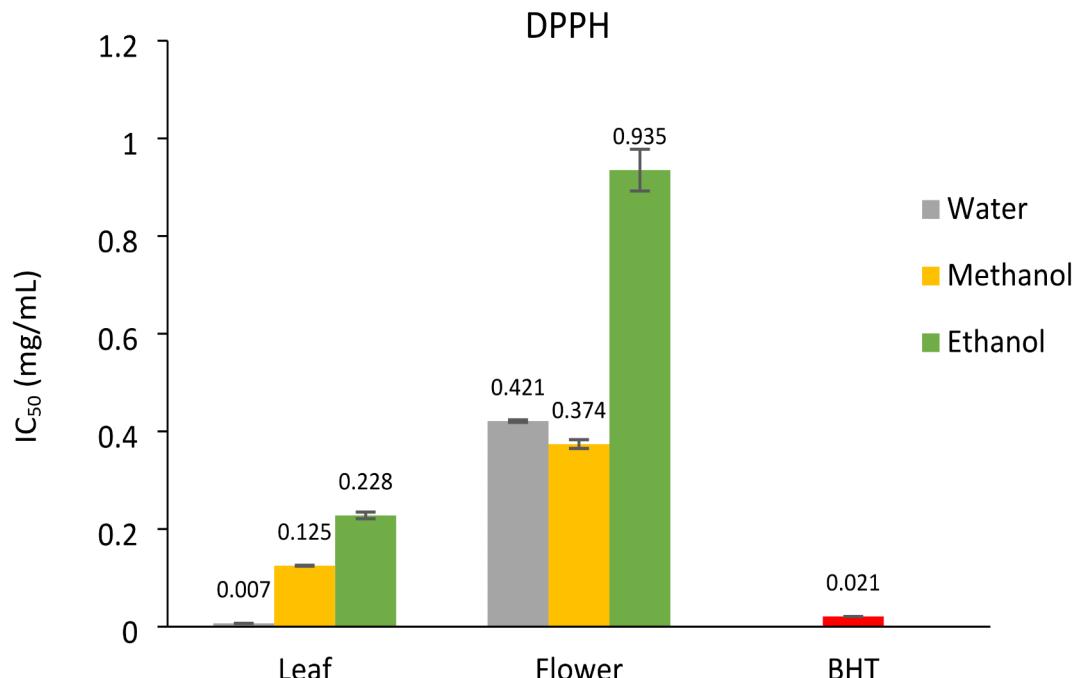


Fig. 2. IC_{50} values (mg/mL) of *A. chamaephaca* antioxidant activity by 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method in water, methanol and ethanol extracts.

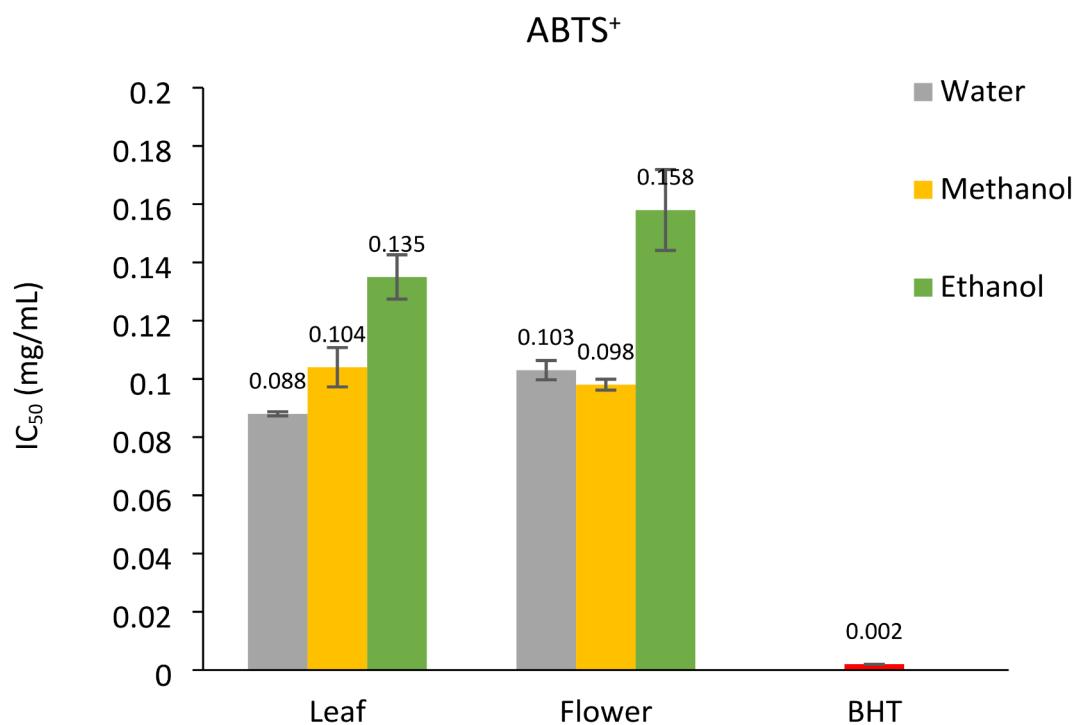


Fig. 3. IC₅₀ values (mg/mL) of *A. chamaephaca* antioxidant activity by ABTS⁺ method in water, methanol and ethanol extracts.

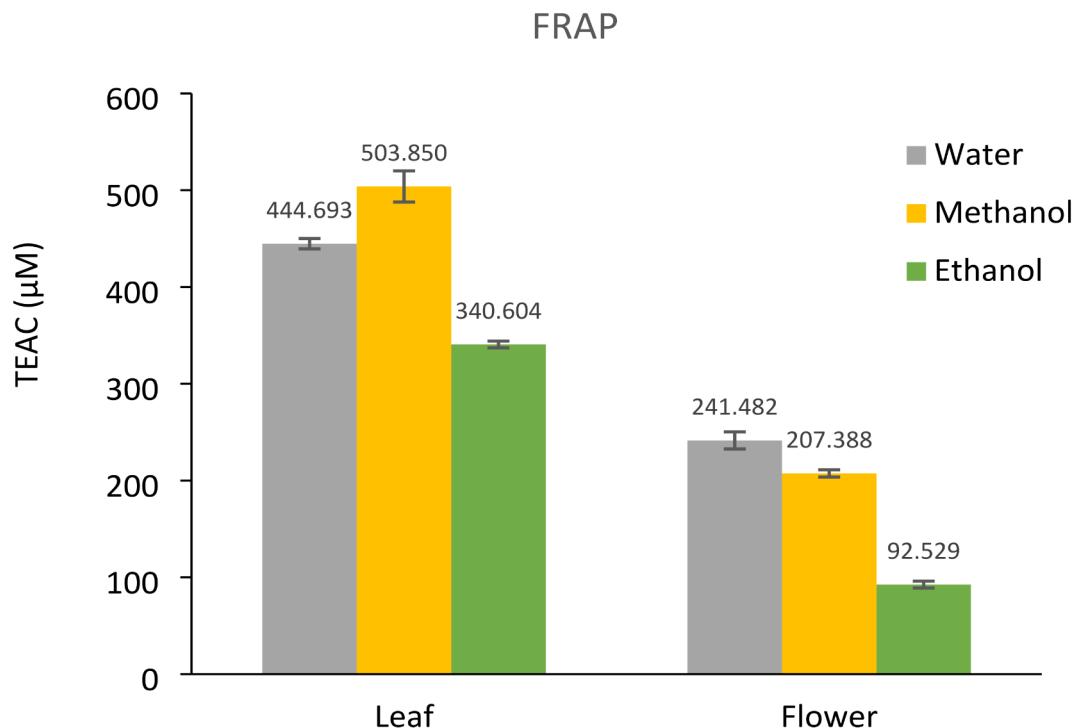


Fig. 4. *A. chamaephaca* antioxidant activity levels by FRAP method in water, methanol and ethanol extracts as μM trolox equivalents (TEAC).

Zhang et al., in their study with *A. complanatus* using methanol by ultrasonic extraction, determined the optimal extraction conditions as 50 °C, 30 min, and 15.1 mL/g. They reported the total phenolic content as 40.12 ± 1.10 mg GAE/g. In the same study, they found a free radical scavenging capacity of 0.85 ± 0.13 mg dry extract/mg DPPH³⁵.

Nayeem et al. determined the total phenolic content in extracts obtained from the stem of *A. spinosus* using methanol and chloroform, with values of 420 and 265 mg GAE/g, respectively. They concluded that the methanol extract of *A. spinosus* exhibited significant dose-dependent analgesic and anti-inflammatory activity³⁶.

Pu et al. conducted studies on total phenols, ABTS⁺, FRAP, and DPPH assays using ethanol, petroleum ether-water, ethyl acetate-water, and n-BuOH-water extractions of *A. taipaishanensis*. Their results indicated that the ethyl acetate-water extraction yielded the best outcomes. They determined the total phenolic content to be 185.45 ± 1.04 mmol equivalent QAE/100 g. The ethyl acetate-water extract demonstrated the highest radical scavenging activity and reducing ability, with ABTS⁺ and FRAP values of 994.50 ± 4.21 μmol Trolox/g and 685.67 ± 3.21 μmol Trolox/g, respectively. Additionally, the DPPH IC₅₀ value was found to be 0.059 ± 0.002 mg/mL. They noted that the main chemical components and bioactivity of *A. taipaishanensis* are similar to those of *A. membranaceus*, a plant recognized as both a medicinal and food source in the pharmacopoeia³⁷.

Naghiloo et al. conducted studies on total phenolic and antioxidant activities in methanol extracts obtained from roots, leaves, and flowers of *A. compactus*. Their results indicated that the extracts from the leaves exhibited the best outcomes, consistent with previous studies on other *Astragalus* species. They determined the total phenol content and DPPH radical scavenging activity in the leaves to be 8.25 μg GAE/mg and 280.5 ± 3.15 g/mL, respectively³⁸.

Arumugam et al. investigated the phenolic profile and antioxidant activity potential of methanol extracts obtained from different parts (flower, stem, leaf and root) of *A. ponticus*. Their findings revealed that the extracts from the leaf showed the best results. They reported that the leaf part of the plant contained a phenolic content of 26.34 ± 0.50 mg GAE/g extract. The DPPH and ABTS⁺ radical scavenging activities from leaf extracts were determined as 43.64 ± 0.94 mg TEs/g extract and 98.79 ± 1.95 mg TEs/g extract, respectively. Also, FRAP reducing power was found to be 56.79 ± 4.34 mg TEs/g extract³⁹.

When comparing the total phenol content, DPPH, FRAP, and ABTS⁺ results from our study with those of different *Astragalus* species in the literature, it was observed that our plant exhibits a medium-level phenol content. The methanol extracts from the leaf part of the plant showed superior DPPH and ABTS⁺ radical scavenging activities, as well as reducing abilities, compared to many species reported in the literature. However, according to the study by Arumugam et al. on the leaf part of *A. ponticus*, this species demonstrated significantly higher radical scavenging activity and reducing ability than our plant.

Mineral analysis

Mineral analysis of the *A. chamaephaca* flower (ACF) and leaf (ACL) parts was conducted using the ICP-OES device, with the results presented in mg/L. The analysis findings are shown in Table 2.

According to the analysis results, the minerals present in the highest concentrations in both parts of the plant are K, Ca, Mg, Fe, and Al. Both parts of the plant are particularly rich in K and Ca. Humans require more than 22 mineral elements, some of which are required in large quantities, while others, such as Se, Cu, Zn, I, and Fe, are required in trace amounts because excessive concentrations can be harmful. Calcium is essential for human health, playing a role in the biological functions of various tissues, including the skeletal system, nervous and heart systems, bones, teeth, and parathyroid glands. Magnesium contributes to muscle and nerve stimulability as the cofactor of up to 300 enzymes. Recent findings suggest that increased magnesium intake can help protect against chronic diseases such as metabolic syndrome, diabetes, cardiovascular diseases, and hypertension. Potassium helps maintain the balance of bodily fluids by transmitting nerve signals and supporting nerve function. The primary function of Fe which is also necessary for energy production is related to myoglobin and hemoglobin synthesis⁴⁰.

In a study conducted by Bronislava et al., the mineral content of *A. glycyphyllos* and *A. cicer* plants was investigated. According to this study, the mineral content in the leaves and flowers of *A. glycyphyllos* was as follows: K (2.22; 2.94 g/100 g), Ca (1.91; 0.463 g/100 g), Mg (0.596; 0.341 g/100 g), P (0.256; 0.428 g/100 g), Zn (2.7; 4.76 mg/100 g), Fe (22.66; 14.10 mg/100 g), respectively. In the mineral analysis of *A. cicer*, the values were:

	Leaf (mg/L)	Flower (mg/L)	LOD	LOQ	R ²	RSD	λ (nm)
Al	$320.53^b \pm 6.09$	$261.53^a \pm 4.97$	0.59	1.80	0.999	1.90	396.1
B	$17.75^b \pm 0.43$	$11.87^a \pm 0.29$	0.62	1.89	0.997	2.42	208.9
Ca	$19769.94^b \pm 351.88$	$7407.43^a \pm 131.85$	1.29	3.91	0.998	1.78	317.9
Fe	147.67 ± 6.20	$429.09^b \pm 18.02$	0.70	2.12	0.998	4.20	259.9
K	$19689.51^b \pm 322.90$	$17680.63^a \pm 289.96$	6.35	19.23	0.995	1.64	766.4
Mg	$706.41^b \pm 17.94$	$604.22^a \pm 15.35$	7.25	21.97	0.990	2.54	279.4
Mn	< LOD	23.15 ± 0.74	0.38	1.14	0.997	3.21	257.6
Na	< LOD	28.15 ± 0.29	3.76	11.41	0.999	1.14	588.9
Zn	$12.30^b \pm 0.16$	$10.33^a \pm 0.13$	2.46	7.46	0.994	1.28	213.8

Table 2. Mineral analysis results (mg/L) of parts of the *A. chamaephaca*.

Phenolic Compound	Part of Plant	Water	Methanol	Ethanol	Phenolic Compound	Part of Plant	Water	Methanol	Ethanol
Gallic Acid	Flower	204.309 ^c ± 9.194	< LOD	< LOD	Rutin	Flower	24.811 ^a ± 0.397	99.917 ^c ± 1.599	70.42 ^c ± 1.127
	Leaf	< LOD	4.113 ^a ± 0.185	10.857 ^b ± 0.489		Leaf	42.346 ^b ± 0.678	76.595 ^d ± 1.226	72.002 ^c ± 1.152
Protocatechuic acid	Flower	10.512 ± 0.515	< LOD	< LOD	Naringenin	Flower	1.126 ^a ± 0.054	12.001 ^b ± 0.576	16.484 ^c ± 0.791
	Leaf	< LOD	< LOD	< LOD		Leaf	< LOD	< LOD	< LOD
Gentisic acid	Flower	< LOD	< LOD	< LOD	Quercetin	Flower	0.540 ^c ± 0.016	6.255 ^e ± 0.181	7.49 ^f ± 0.217
	Leaf	2.842 ± 0.065	< LOD	< LOD		Leaf	0.678 ^d ± 0.012	0.105 ^a ± 0.003	0.160 ^b ± 0.005
Caffeic acid	Flower	38.870 ^c ± 0.972	0.212 ^a ± 0.005	< LOD	Kaempferol	Flower	< LOD	5.937 ^a ± 0.261	6.007 ^a ± 0.264
	Leaf	3.792 ^b ± 0.095	< LOD	< LOD		Leaf	< LOD	< LOD	< LOD
Vannilin	Flower	5.298 ^d ± 0.196	5.944 ^e ± 0.22	3.795 ^c ± 0.14	Taxifolin	Flower	1.552 ^a ± 0.059	5.931 ^c ± 0.225	4.570 ^b ± 0.174
	Leaf	1.488 ^a ± 0.055	2.522 ^b ± 0.093	2.824 ^b ± 0.104		Leaf	< LOD	< LOD	< LOD
Syringaldehyde	Flower	0.314 ^a ± 0.013	0.465 ^b ± 0.019	< LOD	p-Coumaric acid	Flower	233.452 ^d ± 8.171	106.291 ^d ± 3.72	100.306 ^d ± 3.511
	Leaf	< LOD	< LOD	< LOD		Leaf	77.361 ^c ± 2.708	16.439 ^b ± 0.575	11.744 ^a ± 0.411
Ferulic Acid	Flower	27.452 ^d ± 1.235	11.538 ^b ± 0.519	13.652 ^c ± 0.614					
	Leaf	2.021 ^a ± 0.091	< LOD	< LOD					
Salicylic acid	Flower	9.350 ^c ± 0.449	8.203 ^c ± 0.394	3.935 ^b ± 0.189	Sum of Flavonoids	Flower	28.029	130.050	104.971
	Leaf	1.735 ^a ± 0.083	< LOD	< LOD		Leaf	43.024	76.700	72.162
<i>p</i> -Hydroxybenzoic acid	Flower	< LOD	< LOD	< LOD	Sum of Phenolics	Flower	529.557	132.653	121.688
	Leaf	2.237 ^b ± 0.105	0.23 ^a ± 0.011	< LOD		Leaf	91.476	23.304	25.425

Table 3. Results (μg phenolic compound/g dried plant) of phenolic compound (LOD: limit of Detection).

K (2.38; 3.02 g/100 g), Ca (2.09; 0.643 g/100 g), Mg (0.545; 0.328 g/100 g), P (0.288; 0.473 g/100 g), Zn (2.69; 4.61 mg/100 g), Fe (14.52; 13.13 mg/100 g)⁷.

Determination of some phenolic compounds of *A. chamaephaca*

The tendency of each phenol to solubilize, transfer or diffuse into a given solvent is governed by thermodynamics. One of the primary thermodynamic factors describing this tendency is the activity coefficient, which generally affects reversibly the solubility of phenols in non polar solvents (smaller coefficient corresponds to better solubility). For example, in a study by Galanakis et al., ethanol was found to be the most efficient solvent for recovering caffeic acid and its ester derivative, rosmarinic acid. Other phenolic acids, such as ferulic, sinapic, vanillic, and syringic acids, are well soluble in methanol, while gallic acid, cinnamic acid, and coumaric acids are better soluble in water, dichloromethane and acetone, respectively. Many studies have confirmed these differences. Based on this, extractions were carried out using water, methanol and ethanol solvents, which have distinct properties, and the results were evaluated⁴¹.

Twenty-nine different compound screenings were performed to detect phenolic compounds in the plant. The flower part of *A. chamaephaca* was evaluated independently of the solvents; *p*-coumaric acid, gallic acid, rutin, quercetin and Naringenin were detected, respectively (Table 3). In the leaf part of the plant, *p*-coumaric acid, rutin, quercetin, and gallic acid were identified, with *p*-coumaric acid and rutin being the most abundant. Gallic acid, *p*-coumaric acid, and rutin, which were the most abundant compounds detected.

Among these, *p*-coumaric acid, found in the highest amounts in both the flower and leaf parts of *A. chamaephaca*, was evaluated across different solvents. It was primarily found in water (flower = 233.452 μg/g, leaf = 77.361 μg/g dried plant), followed by methanol (flower = 106.291 μg/g, leaf = 16.439 μg/g dried plant) and ethanol (flower = 100.306 μg/g, leaf = 11.744 μg/g dried plant). Numerous studies have focused on *p*-coumaric acid due to its low toxicity, wide natural distribution, and pharmacological effects. It has been shown to act as a neuroprotective agent through its potent antioxidant and anti-apoptotic properties. *p*-coumaric acid can alleviate diabetes symptoms by improving beta-cell function, enhancing glucose transporter (GLUT) expression, increasing antioxidant and anti-inflammatory activities, and modulating enzymes involved in glucose metabolism. Moreover, treatment with *p*-coumaric acid has been found to improve histopathological parameters and reduce kidney damage. In summary, *p*-coumaric acid holds promise as a therapeutic agent with potential applications in the treatment of various diseases⁴².

Gallic acid was detected at a concentration of 204.309 μg/g in the aqueous extract of the flower part of the plant, but it was not found in the other solvent extractions. In the leaf extracts, gallic acid was detected in methanol (4.113 μg/g dried plant) and ethanol (10.857 μg/g dried plant) extracts, but not in the water extract. Gallic acid is known for its wide range of bioactivities, including antioxidant, antimicrobial, anti-inflammatory, and anti-cancer properties. Additionally, anti-HIV, anti-ulcerogenic, and antifungal activities have been reported. Gallic acid is used in various industries, such as a chelating agent in the skin and leather industry first time. It is also employed as a preservative in food and beverages due to its antimicrobial effect⁴³.

Rutin, one of the phenolic compounds, was found in the highest amounts in methanol extracts (flower = 99.917 μg/g dried plant, leaf = 76.595 μg/g dried plant), followed by ethanol (flower = 70.420 μg/g dried plant, leaf = 72.002 μg/g dried plant) and water (flower = 24.811 μg/g dried plant, leaf = 42.346 μg/g dried plant) extracts from both the flower and leaf parts. Due to its strong antioxidant properties, rutin exhibits various biological activities, including anti-inflammatory, antimicrobial, anti-tumor, and anti-asthmatic effects, and

serves as an excellent free radical scavenger. Additionally, rutin is widely used as a stabilizer, preservative, and natural colorant in the pharmaceutical, nutraceutical, and cosmetic industries due to its antioxidative capacity⁴⁴.

While the phenolic compound of Naringenin was detected in the flower part of the plant, it was not detected in any solvent extract of the leaf part. The highest level of Naringenin was found in the ethanol extract (16.484 µg/g dried plant) of the flower part, followed by methanol (12.001 µg/g dried plant) and water (1.126 µg/g dried plant) extracts, respectively. Naringenin has been extensively studied for its anti-diabetic activity, where it has been shown to reduce blood glucose levels. Naringenin also inhibits the proliferation of cancer cells across a broad range of cancer types, including colon, breast, stomach, prostate, liver, cervix, pancreas, uterus, and leukemia. The antioxidant and anti-inflammatory mechanisms of Naringenin contribute to its neuroprotective effects. Beyond hyperlipidemia and atherosclerosis, numerous studies have highlighted its protective role in various cardiovascular diseases⁴⁵.

Quercetin was found in the flower part of the plant in ethanol (7.490 µg/g dried plant), methanol (6.255 µg/g dried plant) and water (0.540 µg/g dried plant) extracts, and in the leaf part in water (0.678 µg/g dried plant), ethanol (0.160 µg/g dried plant) and methanol (0.105 µg/g dried plant) extracts, respectively. Kaempferol, another phenolic compound, was detected in the flower part but not in any solvent extract from the leaf part. The highest concentration of kaempferol was detected in the ethanol extract (6.007 µg/g dried plant) in the flower part, followed by methanol (5.937 µg/g dried plant), with no detection in the water extract. Flavonoids, particularly kaempferol and quercetin, are among the bioactive compounds that contribute significantly to human health. Both kaempferol and quercetin offer heart-protective and antihypertensive benefits. In addition, they exhibit antifungal and bacterial properties. In recent years, the significance of these flavonoids has increased due to their wide range of beneficial bioactive effects, such as anti-viral, anti-bacterial, antifungal, anti-inflammatory and cardioprotective properties⁴⁶.

In the study conducted by Albayrak et al., the phenolic compositions of the *Astragalus* extracts were analyzed using LC-MS. They identified chlorogenic acid, epicatechin, catechin hydrate, rutin, quercetin, kaempferol, syringic acid, cinnamic acid and ferulic acid. Among these compounds, ferulic acid was found to be the most abundant, with a concentration of 1123.9 µg/g dried plant, followed by syringic acid (735.18 µg/g dried plant) and cinnamic acid (558 µg/g dried plant). The compound detected in the least amount was quercetin (293.5 µg/g dried plant)⁴⁷.

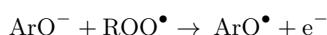
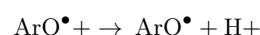
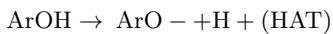
Phenolic acids generally have one aromatic ring, along with carboxyl and hydroxyl groups in their structure. These groups increase the polarity of the molecule, making phenolic acids highly soluble in water²⁸. In contrast, although flavones contain hydroxyl groups, the increased number of aromatic rings with apolar properties reduces the overall polarity of the molecules²⁸. This results in lower solubility in highly polar solvents like water²⁸. Methanol and ethanol solvents have been reported to dissolve flavone molecules better because they are less polar than water²⁸. According to the findings of this study, Gallic acid, Caffeic acid, Protocatechuic acid phenolic compounds were detected in higher amounts in water solvent, while Rutin, Naringenin, Quercetin and Kaempferol flavonoid compounds were detected in higher amounts in methanol / ethanol solvents.

Free radicals are atoms, molecules or ions with unpaired electrons that are highly unstable and active towards chemical reactions with other molecules. They derive from three elements: oxygen, nitrogen and sulfur. For example, oxygen-centered free radicals are known as reactive oxygen species (ROS) and include superoxide (O_2^-), hydroxyl ($HO\cdot$), peroxy (ROO \cdot), alkoxy (RO \cdot) and nitric oxide ($NO\cdot$). The hydroxyl (half-life of 10^{-9} s) and the alkoxy (half-life of seconds) free radicals are very reactive and rapidly attack the molecules in nearby cells, and probably the damage caused by them is unavoidable and is dealt with by repair processes⁴⁸⁻⁵⁰.

Antioxidants play a vital role in both food systems as well as in the human body to reduce oxidative processes and harmful effects of ROS^{51,52}.

The antioxidant potential of phenolic compounds depends on the number and arrangement of the hydroxyl groups in the molecules of interest. The antioxidant activity of phenolic acids and their derivatives depends on the number and position of the hydroxyl groups bound to the aromatic ring, the binding site and mutual position of hydroxyl groups in the aromatic ring, and the type of substituents⁵³⁻⁵⁵. Substitution in phenolic compounds at the meta-position has a rather limited effect. Steric and electronic effects are responsible for the antioxidant activities and stoichiometric factors of the chain-breaking phenolic antioxidants⁵⁶. For elucidation of the hydrogen abstraction mechanism of phenolic antioxidants in the chain process of autoxidation, molecular orbital theory has been applied^{55,57}.

In radical scavenging studies, the antioxidant effects of phenolic compounds (Ar-OH) generally occur by two mechanisms, including hydrogen atom transfer (HAT) or single-electron transfer followed by proton transfer (SET-PT). However, in some cases, it may not be possible to separate these two mechanisms with clear boundaries⁵⁸. In a HAT-based assay, an antioxidant molecule can quench free radicals through H-donation, while in a SET-based method, a potential antioxidant agent exhibits antioxidant ability by transferring an electron (e $^-$) to reduce any compound, including radicals, metals, and carbonyls⁵⁹. Recently, in addition to these two mechanisms, a third mechanism called the sequential proton loss electron transfer (SPLET) mechanism has been developed.





The -OH group in the 7th position of flavonoids had great importance as the site of ionization and electron transfer, according to SPLET. This mechanism has been discovered recently^{60,61}. In the first step, the reaction enthalpy corresponds to the proton affinity of the phenoxide anion (ArO^-), while in the second step, the phenoxy radical is formed by electron transfer from the phenoxide anion to ROO^\bullet . In terms of antioxidant effect, SPLET is similar to free radicals in the HAT mechanism.

In addition, since the SET-PT and SPLET mechanisms in the solvent environment are important, the effect of water on the three mechanisms also has significance. Additionally, Litwinienko and Ingold (2005) proposed a different mechanism for SPLET. Among organic solvents, methanol is the leading solvent that supports ionization. This mechanism is preferred in that phenols with low pK_a react with electron-deficient radicals with relatively lower HAT activities and yield product molecules with low pK_a ⁶⁰. In addition, SPLET formation in methanol and ethanol solutions was also reported by Foti et al.⁶². Many studies have shown that DPPH[•] reacts with phenolic acids. As a result of the suppression of the ionization of the phenolic hydroxyl group by the free carboxylic acid, the rate constants of the reactions for methyl esters of these acids are several times higher than for free acids. These experiments nicely confirm the effective role of ionization of phenolic compounds in the reaction of phenols with DPPH[•] in solvents that can promote ionization^{53,61}.

The findings obtained from the study suggest that the identification and isolation of specific compounds with high antioxidant activity, particularly from methanol and water extracts, could contribute to research on the mechanisms of underlying antioxidant activity.

Conclusion

In the study, the leaf and flower parts of *A. chamaephaca* were separated and extracted using water, methanol, and ethanol. The total phenolic content, measured using the Folin-Ciocalteu method, showed the highest value in the methanol extracts from the leaf part. The flower part exhibited the lowest phenolic compound levels across all extracts.

The water extract from the leaf of *A. chamaephaca* demonstrated the highest DPPH antioxidant activity, while the ethanol extract showed the lowest activity. When comparing the IC₅₀ values of BHT and the plant extracts, the water extract of the leaf exhibited a higher antioxidant capacity than BHT. In the FRAP method, the highest antioxidant capacity was observed in the methanol extract of the leaf, while the lowest FRAP activity was found in the ethanol extract of the flower. In the ABTS⁺ free cation radical scavenging method, the highest activity in the leaf was detected in the water extract, whereas the highest activity in the flower was found in the methanol extract. The lowest ABTS⁺ values were determined in the ethanol extracts of the plant.

LC-MS/MS was employed to screen 29 different compounds for the detection and determination of phenolic compounds in all extracts. High levels of p-coumaric acid, gallic acid, rutin, quercetin, and naringenin were detected in the flower and leaf parts of *A. chamaephaca*, irrespective of the solvent used. The highest phenolic components in the leaf part were p-coumaric acid, rutin, and gallic acid. p-Coumaric acid and gallic acid, known for their numerous pharmacological effects, were predominantly found in the water extract of the flower part. Naringenin was detected only in the flower part and was absent in any solvent extract of the leaf part. Rutin was found in the highest amounts in the methanol extracts, while kaempferol was predominantly detected in the ethanol extract of the flower part. Quercetin was identified in all plant extracts through LC-MS/MS analysis.

According to the ICP-OES analyses, K, Ca, B, Zn, Mg, Fe and Al were detected in both parts of the plant, with K and Ca being the most abundant. These detected elements include both the major and minor minerals essential for human health.

In general, the potential health-promoting properties of *A. chamaephaca*, especially the presence of antioxidant-rich leaf extracts and bioactive phenolic compounds with various biological activities were highlighted by comprehensive analysis. These findings offer valuable insights for further investigation of *A. chamaephaca* as a potential source of natural antioxidants and bioactive compounds with therapeutic applications in various health conditions. However, further research is required to explore the underlying mechanisms of action and confirm the therapeutic efficacy of these bioactive components *in vivo*.

Phenolic substances identified in this study are compounds of significant industrial value across various fields, such as food, cosmetics and medicine. For this reason, the *Astragalus* genus also has the potential to be used in various industrial areas.

Data availability

The datasets used and analyzed during the current study available from the corresponding author on reasonable request.

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Author contributions

Hacer Doğan: Analysis, Investigation, Resources, Writing -review & editing. Ömer Kayır: Analysis, Investigation, Resources, Writing -review & editing. Ömer Koray Yaylacı: The collected herbs were identified by him. İbrahim Bilici: Supervision, collecting, Methodology, Investigation, Resources, Writing -review & editing. Erol Alver: Supervision, collecting, Methodology, Investigation, Resources, Writing.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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