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ARAŞTIRMA MAKALESİ RESEARCH ARTICLE

Phytochemical Characterization and Preliminary In Vitro Cytotoxic Activity of the Endemic Plant *Cousinia birecikensis Hub.-Mor.**

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Abstract

Aim: This study aimed to characterize the phytochemical composition of the endemic plant Cousinia birecikensis and to evaluate its antioxidant capacity and preliminary in vitro cytotoxic activity.

Materials and Methods: Sequential extracts (hexane, dichloromethane, methanol, and aqueous) were prepared. Total phenolic and flavonoid contents were determined, and antioxidant capacity was evaluated using DPPH, ABTS, FRAP, and CUPRAC assays. LC-MS/MS analysis was performed for phytochemical profiling. Cytotoxic effects were assessed using the MTT assay on A549 and BEAS-2B cell lines, and apoptosis was evaluated using Annexin V-FITC/PI staining.

Results: Methanol and aqueous extracts exhibited higher phenolic content and antioxidant activity ($p<0.05$). LC-MS/MS analysis revealed fumaric acid and vanillic acid as major compounds. The methanol extract showed dose-dependent cytotoxic activity on A549 cells (IC^{50}: 89.67 µg/mL), while lower toxicity was observed in BEAS-2B cells. Annexin-V analysis indicated increased apoptotic cell populations.

Conclusion: The findings demonstrate that *Cousinia birecikensis* possesses significant antioxidant capacity and preliminary in vitro cytotoxic activity. Further in vivo and mechanistic studies are required to validate these findings.

Keywords: *Cousinia birecikensis, Antioxidant, Biochemical contents, Cytotoxic activity, Nutraceutical*

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Endemik Bitki Cousinia birecikensis'in Fitokimyasal Karakterizasyonu ve Ön İn Vitro Sitotoksik Aktivitesi

Özet

Amaç: Bu çalışma, endemik bitki *Cousinia birecikensis*'in fitokimyasal bileşimini karakterize etmeyi ve antioksidan kapasitesini ve ön in vitro sitotoksik aktivitesini değerlendirmeyi amaçlamıştır.

Gereç ve Yöntem: Ardışık ekstraktlar (heksan, diklorometan, metanol ve sulu) hazırlandı. Toplam fenolik ve flavonoid içerikleri belirlendi ve antioksidan kapasitesi DPPH, ABTS, FRAP ve CUPRAC testleri kullanılarak değerlendirildi. Fitokimyasal profilleme için LC-MS/MS analizi yapıldı. Sitotoksik etkiler, A549 ve BEAS-2B hücre hatlarında MTT testi kullanılarak değerlendirildi ve apoptoz, Annexin V-FITC/PI boyama yöntemi kullanılarak değerlendirildi.

Bulgular: Metanol ve sulu ekstraktlar daha yüksek fenolik içerik ve antioksidan aktivite sergiledi ($p<0,05$). LC-MS/MS analizi, fumarik asit ve vanilik asidin ana bileşikler olduğunu ortaya koydu. Metanol ekstresi, A549 hücrelerinde doza bağımlı sitotoksik aktivite gösterdi (IC^{50}: 89,67 µg/mL), BEAS-2B hücrelerinde ise daha düşük toksisite gözlendi. Annexin-V analizi, apoptotik hücre popülasyonlarında artış olduğunu gösterdi.

Sonuç: *Cousinia birecikensis*'in önemli antioksidan kapasiteye ve ön in vitro sitotoksik aktiviteye sahip olduğunu göstermektedir. Bu bulguları doğrulamak için daha ileri in vivo ve mekanistik çalışmalara ihtiyaç duyulmaktadır.

Anahtar Kelimeler: *Cousinia birecikensis, Antioksidan, Biyokimyasal içerikler, Sitotoksik aktivite, Nutrasötik*

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Abbreviations: TPC: Total Phenolic Content, **TFC:** Total Flavonoid Content, **DPPH:** 1,1-Diphenyl-2-picrylhydrazyl, **ABTS:** 2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid), **FRAP:** Ferric Reducing Antioxidant Power, **CUPRAC:** Cupric Ion Reducing Antioxidant Capacity, **GAE:** gallic acid equivalent, **QE:** quercetin equivalent, **TE:** Trolox equivalent

INTRODUCTION

Plants play a central role in both modern and traditional medical practices and are at the forefront of pharmacological research. The phytochemicals they contain serve as a rich reservoir of molecular structures with healing properties. In addition to their contributions to the management and treatment of various diseases, these plants are also valuable components of defense mechanisms developed against global health challenges (1).

When examined from a technical perspective in the literature, medicinal plants are defined as biological resources that, thanks to the pharmacologically active molecules they contain, offer the possibility of therapeutic intervention—either directly or indirectly—in the prevention or treatment of specific pathologies (2).

Numerous studies aimed at determining the pharmacological potential of plants have examined the medical characteristics of these natural sources across a wide range, from their anti-inflammatory effects to their antitumor properties, and from their ability to combat diabetes to their components that combat oxidative stress (3).

Antioxidants are molecules that sacrifice themselves in the reduction process of free radicals, thereby oxidizing themselves and protecting neutral substrates. To halt the oxidation of biological structures, this defense mechanism must enter into a rapid reaction cycle with free radicals. The radical form resulting from the oxidation of the antioxidant must generate stable derivatives to disrupt the radical production mechanism within the system (4).

When the body's natural enzymatic defense cannot completely neutralize the radical species produced, oxidative stress develops. Identifying new antioxidant sources to bolster these defense mechanisms is of great importance in this context. Today, a wide variety of analytical methods are utilized to determine the efficacy of these potential sources and manage their development processes. In general, antioxidant compounds that enhance the body's resistance to free radicals are often obtained externally through dietary intake (5).

Pharmacologically active compounds isolated from natural sources play a strategic role in chemotherapeutic processes due to their greater compatibility with human physiology and relatively low side effect profiles. These plant-derived agents stand out for their potential to minimize the severe complications associated with conventional treatment methods (6).

Extracts derived from taxa belonging to the Asteraceae (Compositae) family exhibit a strong radical scavenging capacity due to their phenolic compound composition and other bioactive substances. These phenolic derivatives demonstrate therapeutic efficacy by stimulating endogenous antioxidant defense mechanisms, inactivating transition metal ions through chelation, and inhibiting free radical production pathways (7).

The primary objective of this study is to characterize the pharmacological properties of the endemic plant *Cousinia birecikensis* (*C. birecikensis*) (Asteraceae) is a species native to Türkiye and represents a valuable source of bioactive compounds. During the study, the antioxidant potential of the plant extracts (using ABTS, CUPRAC, FRAP, and DPPH methods), their metabolomic content (amino acids, carnitine, and vitamins), the structure of phenolic compounds, and trace element levels were characterized in detail. Additionally, the plant's in vitro activity against cancer cell lines was tested.

MATERIALS AND METHODS

Collection of Plants and Preparation of Solutions from Plant Samples

C. birecikensis plant material was collected from its natural habitats in the Atatürk Dam Lake basin (Euphrates River) within the province of Şanlıurfa. The plants were collected during the period when the taxon was most suitable for pharmacological analysis. After the *C. birecikensi*s plant samples used in the study were collected, they were left to dry in a shaded environment, away from direct sunlight, to preserve their phytochemical content. The dried plant material (flowers, leaves, and roots) was ground into a homogeneous powder using a mechanical grinder.

Portions of 100 grams were weighed from the powdered samples and, in the first stage, left to macerate in hexane at room temperature overnight. Following the incubation period, the mixtures were homogenized using an ultrasonicator for 15 minutes to enhance extraction efficiency. The resulting suspensions were filtered using Whatman filter paper, and the filtrate was concentrated using a rotary evaporator to remove the solvent, yielding the crude extract.

The plant residues separated from the solvent were dried in a freeze-dryer in preparation for the next step. The same extraction procedure was repeated using dichloromethane, methanol, and distilled water as solvents, in that order. Thanks to this stepwise solvent system, four distinct fractions with varying levels of polarity (hexane, dichloromethane, methanol, and aqueous) were obtained from the plant.

Total phenolic content determination

The total amount of phenolic compounds in the extracts was determined spectrophotometrically using the Folin-Ciocalteu reagent in accordance with the established methodology (8). Gallic acid was selected as the standard for quantitative analysis in this study. The absorbance values of the prepared standard series and samples were measured at a wavelength of 760 nm against a blank solution using a spectrophotometer. The obtained data were calculated using the gallic acid standard curve.

Total Flavonoid Content Determination

The total flavonoid content of the samples was determined using a colorimetric method (9). The intensity of the resulting chromogenic complex is directly proportional to the flavo-

noid concentration of the sample and was analyzed spectrophotometrically at a wavelength of 510 nm. The obtained absorbance data were calculated using a calibration curve established with quercetin as the standard.

FRAP Method (Ferric Reducing Antioxidant Power Analysis)
To determine the total reducing capacities of the extracts, fresh FRAP reagent was first prepared during the analysis process. A homogeneous mixture was obtained by adding 1 mL of plant extract and 2 mL of FRAP reagent to test tubes. The mixtures were incubated at room temperature for 30 minutes to allow the reaction to complete. At the end of the incubation period, the color intensity of the formed complex was measured spectrophotometrically at a wavelength of 593 nm (10, 11). Troloks was used as the standard for quantitative evaluation, and the entire procedure was repeated for the standard series.

Determination of ABTS Radical Scavenging Activity
The ABTS radical scavenging capacities of the extracts were determined by measuring the absorbance values of the samples and standard solutions spectrophotometrically at a wavelength of 734 nm. As a control group, a reaction mixture containing pure solvent (methanol) instead of plant extract was used (12). The radical scavenging percentage for each sample was calculated using the following formula:

ABTS (%) = [(C0-C1) / C0] x 100
C0 = Control absorbance value
C1 = Absorbance value of the sample and standard

Concentration-activity curves were plotted based on the calculated percentage inhibition values for the plant extracts and standards. The resulting curves were correlated with data from Troloks, which was used as a standard, and the results were reported in terms of Troloks equivalents.

CUPRAC method
To determine the copper-reducing antioxidant capacities of the extracts, 40 µL of a plant extract sample was added to the prepared reagent mixture and incubated at room temperature for 30 minutes. At the end of the incubation period, the absorbance values of the resulting colored complex were measured spectrophotometrically at a wavelength of 450 nm. Concentration-absorbance curves were plotted using the data obtained for both plant extracts and standard solutions, and the total reducing capacities of the samples were calculated (13,14).

DPPH Method
The free radical scavenging capacities of the extracts were determined using the stable DPPH radical. As part of the analysis, the absorbance values of the samples and standard solutions were measured spectrophotometrically at a wavelength of 517 nm (15). The radical scavenging activities of the samples were calculated as a percentage of inhibition using the following formula, compared to the negative control:

DPPH (%) = [(C0-C1) / C0] x 100
C0 = Control absorbance value
C1 = Absorbance value of the sample and standard

Concentration-activity graphs were constructed using the percentage values obtained for the plant extracts and standards. The data obtained from these graphs were correlated with the Troloks curve used as a standard, and the results were quantified in Troloks equivalent units.

Content Analyses of Plant Extracts
Advanced analytical techniques were employed to determine the phytochemical composition of methanol extracts obtained from the aboveground parts of the *C. birecikensis* plant. In this context, the phenolic compound profile, free amino acid content, and carnitine levels of the extracts were characterized using a high-sensitivity Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS) system.

The trace element content of the samples was determined using the Inductively Coupled Plasma (ICP) method, a plasma-based ionization technique. Additionally, the concentrations of glucose, vitamin B12, and folic acid—which are of critical biochemical importance—were

quantified using the chemiluminescence method, which provides high specificity. This integrated analytical approach enabled the comprehensive mapping of both the organic and inorganic components of the plant extract.

Trace Element Content Analysis

The Inductively Coupled Plasma (ICP) method, a highly sensitive analytical technique, was used to determine the trace element composition in plant extracts. The ICP method is considered the gold standard in trace element analysis due to its low limits of detection (LOD), wide dynamic measurement range, and ability to simultaneously quantify multiple elements (multielemental analysis). In this study, the quantification of metals and metalloids in the extracts was performed within the framework of the high reproducibility and accuracy parameters provided by this method.

Investigation of the cytotoxic Activities of Plant Extracts

Cell culture

We would like to inform you that the cell lines used in this study are sourced and certified by the American Type Culture Collection (ATCC), and therefore do not require ethical committee approval. In this study, lung cancer epithelial (A549) and normal lung epithelial (BEAS-2B) cell lines were used. The cell lines used in the study were cultured by thawing samples stored under cryopreservation (stock) conditions in liquid nitrogen. For cell maintenance, appropriate culture media (RPMI-1640) enriched with 10% Fetal Bovine Serum (FBS) and supplemented with streptomycin and penicillin to prevent contamination were used.

Following the incubation process, cells that reached appropriate confluence (surface coverage density) on the plate surface within approximately 24 hours were detached from the surface using trypsinization, an enzymatic method. After centrifugation and counting, the cells were seeded onto plates at appropriate densities for the experimental groups and prepared for in vitro analysis.

Determination of cytotoxic effects using the MTT assay

The effects of plant extracts on cell viability were determined using the MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay, which is based on mitochondrial metabolic activity. The basic principle of this method is that succinate dehydrogenase enzymes present in the mitochondria of live cells reduce the yellow-colored tetrazolium salt, converting it into purple-colored, water-insoluble formazan crystals. As part of the experimental procedure, normal and cancer cells in the logarithmic growth phase were seeded into 96-well microplates at a density of 1×10^4 cells per well and incubated for 24 hours. Following incubation, the existing culture medium was removed, and extracts at varying concentrations ranging from 0 to 200 µg/ml were applied to the cells. Wells containing only culture medium and no other substances were designated as "blank," while wells containing only cells and culture medium were designated as the "negative control" group. After a 24-hour incubation following the application, 10 µL of MTT solution was added to each well, and the plates were incubated in the dark for 4 hours. At the end of the incubation period, 100 µL of DMSO was added to the wells to dissolve the formed formazan crystals. The absorbance values of the resulting colored complex were measured at a wavelength of 570 nm using a microplate reader. Assuming the viability of the control group was 100%, the relative cell viability percentages (%) were calculated based on the absorbance values of the samples. Cell viability ratios in the experimental groups were calculated based on the normalization of the optical density (OD) data obtained from spectrophotometric analyses relative to the control group. Accordingly, the relative cell viability (viability) was determined by dividing the absorbance value of each sample by the average absorbance of the untreated control group and then converting it to a percentage:

([{Sample OD} / {Control OD}] × 100). These percentage values were used to quantitatively demonstrate the inhibitory effect of *C. birecikensis* extracts on cell proliferation and their cytotoxic potential.

Annexin V-FITC/PI flow cytometry analysis

The determination of cell death pathways (ap-

optotic or necrotic) was performed using the Annexin V-FITC and Propidium Iodide (PI) double-staining method. The principle of this method is based on the detection of Annexin V-FITC, a calcium-dependent fluorescent dye that binds to phosphatidylserine (PS) molecules, which translocate from the inner to the outer surface of the cell membrane during the early stages of apoptosis. FITC generates green fluorescence in cells bound to Annexin V, thereby identifying the early apoptotic population.

To identify dead or late apoptotic/necrotic cells, the PI dye was used, which intercalates into the nucleic acids of cells with disrupted membrane integrity and emits red fluorescence. The cells were analyzed using flow cytometry based on their fluorescence intensity and classified into four main populations: live, early apoptotic, late apoptotic, and necrotic.

Statistical Analysis

All experiments were performed in triplicate (n=3) and results are presented as mean ± standard deviation (SD). Statistical analyses were carried out using GraphPad Prism software (version XX). Differences between groups were evaluated using one-way ANOVA followed by Tukey's post hoc test. A *p*-value <0.05 was considered statistically significant.

IC_{50} Calculation

The half-maximal inhibitory concentration (IC_{50}) values were determined using nonlinear regression analysis (log(inhibitor) vs. normalized response) in GraphPad Prism. The SC_{50} values (the concentration required to scavenge 50% of free radicals) for DPPH and ABTS assays were calculated using nonlinear regression analysis based on concentration–inhibition curves. GraphPad Prism software was used for these calculations.

Solvent Control

All extract solutions were prepared in DMSO, and the final DMSO concentration in cell culture did not exceed 0.1%. A vehicle control group containing the same concentration of DMSO was included in all experiments.

RESULTS

Total phenolic content determination

For the determination of total phenolic content (TPC) in plant extracts, a calibration curve was established using gallic acid as the standard. The phenolic content values calculated based on the obtained data were standardized in mg GAE/g. When *C. birecikensis* flower extracts were analyzed, the most pronounced TPC values were observed in the methanol fraction, followed by the dichloromethane, water, and hexane extracts, respectively (183.06 ± 9.15; 52.3 ± 2.61; 51.95 ± 2.59; 31.95 ± 1.59 mg GAE/g). In leaf extracts, the hierarchy was methanol > water > dichloromethane > hexane, with numerical values recorded as 88.55 ± 4.42; 80.56 ± 4.02; 12.44 ± 0.62; and 12.02 ± 0.60 mg GAE/g, respectively. Similarly, in root extracts, the highest phenolic content was observed in the methanol phase (133.06 ± 6.65 mg GAE/g), while the efficiency of the other solvents was ranked as water (79.45 ± 3.97), dichloromethane (29.04 ± 1.45 mg GAE/g), and hexane (3.69 ± 0.18 mg GAE/g). The TPC results of the extracts are shown in Table 1.

Total flavonoid content determination

The total flavonoid contents (TFC) of the analyzed plant extracts were determined using a linear regression equation established with quercetin as the standard. The results are expressed in mg quercetin equivalent (mg QE/g). When the flavonoid contents of *C. birecikensis* flower extracts were examined, the highest values were observed in the methanol fraction (528.44 ± 26.42 mg QE/g). This was followed, in order, by the dichloromethane (261.08 ± 13.05 mg QE/g), water (25.52 ± 1.27 mg QE/g), and hexane (24 ± 1.2 mg QE/g) extracts.

In leaf samples, however, the hierarchy changed depending on solvent polarity; with the highest content detected in the water extract (166.63 ± 8.33 mg QE/g), while methanol (140.25 ± 7.01 mg QE/g), dichloromethane (33.16 ± 1.65 mg QE/g), and hexane (19.97 ± 0.99 mg QE/g) exhibited lower concentrations. In the data obtained from root extracts, the methanol phase (197.33 ± 9.86 mg QE/g) stood out; the water, dichloromethane, and hexane extracts, on the

other hand, completed this ranking with values of 163.86 ± 8.19; 132.61 ± 6.63, and 13.3 ± 0.66 mg QE/g, respectively. The TFC results of the extracts are shown in Table 1.

Table 1. Total phenolic content and total flavonoid content of *C. birecikensis* extracts

Plant part	Solvent	TPC (mg GAE/g)	TFC (mg QE/g)
Flowers	Water	51.95 ± 2.59	25.52 ± 1.27
	Dichloromethane	52.30 ± 2.61	261.08 ± 13.05
	Hexane	31.95 ± 1.59	24.00 ± 1.20
	Methanol	183.06 ± 9.15	528.44 ± 26.42
Leaves	Water	80.56 ± 4.02	166.63 ± 8.33
	Dichloromethane	12.44 ± 0.62	33.16 ± 1.65
	Hexane	12.02 ± 0.60	19.97 ± 0.99
	Methanol	88.55 ± 4.42	140.25 ± 7.01
Roots	Water	79.45 ± 3.97	163.86 ± 8.19
	Dichloromethane	29.04 ± 1.45	132.61 ± 6.63
	Hexane	3.69 ± 0.18	13.30 ± 0.66
	Methanol	133.06 ± 6.65	197.33 ± 9.86

Values are expressed as mean ± standard deviation (SD) of three independent experiments (n = 3).

DPPH assay

The free radical scavenging potentials of the extracts were determined using the DPPH method, and their antioxidant capacities were calculated in terms of the standard Trolox equivalent (mg Trolox/g). The strongest antioxidant activity observed in *C. birecikensis* flower extracts was detected in the methanol fraction (SC_{50} 45.66 ± 4.38 µg/mL), followed by the hexane, dichloromethane, and water extracts, which exhibited values of 67.06 ± 6.20; 62.17 ± 6.10, and 55.66 ± 5.88 µg/mL, respectively.

When the findings from leaf samples were examined, the SC_{50} values were as follows: methanol (56.38 ± 4.31 µg/mL), water (55.46 ± 4.27 µg/mL), hexane (64.85 ± 5.24 µg/mL), and dichloromethane (63.21 ± 5.16 µg/mL) extracts showed a similar yet hierarchical activity pattern. A similar pattern was observed for root extracts, with the highest scavenging effect SC_{50} recorded in the methanol extraction (52.44 ± 3.32 µg/mL). The other activity values for the root region were ranked as follows: water (54.06 ± 4.20 µg/mL), dichloromethane (62 ± 5.20 µg/mL), and hexane (68.51 ± 6.17 µg/mL). The DPPH results of the extracts are shown in Table 2.

ABTS assay

Troloks was used as the standard in the ABTS radical scavenging activity assay conducted as part of this study. When the antioxidant capacities of extracts obtained from different parts of the *C. birecikensis* plant were examined, the order of activity in the flower extracts was methanol > water > dichloromethane > hexane (SC_{50} 40.03 ± 3.80; 45.94 ± 4.74; 58.68 ± 5.43; 160.1 ± 12.80 µg/mL).

In leaf extracts, the highest activity values were observed in the water fraction, followed by methanol, dichloromethane, and hexane (SC_{50} 45.69 ± 4.78; 47.26 ± 5.71; 84.67 ± 9.23; 112.83 ± 11.64 µg/mL, respectively). Similarly, in root extracts, solvents with higher polarity (water and methanol) were found to exhibit stronger antioxidant potential (SC_{50} 42.48 ± 3.77; 45.26 ± 3.91; 77.05 ± 6.85; 124.94 ± 11.74 µg/mL). The ABTS results of the extracts are shown in Table 2.

FRAP assay

The iron-reducing antioxidant capacity (FRAP) of the extracts was determined using the standard compound Trolox (mg TE/g). As a result of the analyses, the antioxidant potential of *C. birecikensis* flower extracts was determined using methanol, dichloromethane, water, and hexane solvents in that order (1061.96 ± 53.09; 543.39 ± 27.16; 332.14 ± 16.60; 49.82 ± 2.49

mg TE/g). In leaf samples, the most pronounced activity was measured in the water extract, followed by the methanol, dichloromethane, and hexane fractions (507.85 ± 25.39; 472.85 ± 23.64; 117.5 ± 5.87; 41.07 ± 2.05 mg TE/g). In root extracts, the highest FRAP values were obtained in the methanol and water extracts, while dichloromethane and hexane exhibited lower capacity (683.75 ± 34.18; 641.07 ± 32.05; 196.96 ± 9.84; 42.5 ± 2.12 mg TE/g). The FRAP results of the extracts are shown in Table 2.

CUPRAC assay

The copper (II) reducing antioxidant capacities (CUPRAC) of the extracts were determined using analyses conducted with Troloks (mg TE/g) as the standard. The hierarchy of antioxidant activity observed in *C. birecikensis* flower extracts showed a sequence of methanol, water, dichloromethane, and hexane (202.82 ± 11.14; 127.16 ± 6.35; 36.3 ± 1.81; 32.75 ± 1.63 mg TE/g).

Upon examining the findings from leaf samples, it was determined that the highest values were again concentrated in the methanol extract, followed by the water, dichloromethane, and hexane fractions (200.12 ± 10.03; 143.82 ± 9.12; 48.04 ± 2.40; 17.6 ± 0.88 mg TE/g). A similar trend was observed in root extracts, with the strongest reducing capacity measured in the methanol fraction (176.22 ± 9.18 mg TE/g), while the lowest activity was detected in the hexane extract (11.22 ± 0.56 mg TE/g). The CUPRAC results of the extracts are shown in Table 2.

Table 2. Antioxidant activity results of *C. birecikensis* extracts

Plant part	Solvent	DPPH SC_{50} (µg/mL)	ABTS SC_{50} (µg/mL)	FRAP (mgTrolox/g)	CUPRAC (mgTrolox/g)
Flowers	Water	55.66 ± 5.88	45.94 ± 4.74	332.14 ± 16.60	127.16 ± 6.35
	Dichloromethane	62.17 ± 6.10	58.68 ± 5.43	543.39 ± 27.16	36.30 ± 1.81
	Hexane	67.06 ± 6.20	160.10 ± 12.80	49.82 ± 2.49	32.75 ± 1.63
	Methanol	45.66 ± 4.38	40.03 ± 3.80	1061.96 ±53.09	202.82 ± 11.14
Leaves	Water	55.46 ± 4.27	45.69 ± 4.78	507.85 ± 25.39	143.82 ± 9.12
	Dichloromethane	63.21 ± 5.16	84.67 ± 9.23	117.50 ± 5.87	48.04 ± 2.40
	Hexane	64.85 ± 5.24	112.83 ± 11.64	41.07 ± 2.05	17.60 ± 0.88
	Methanol	56.38 ± 4.31	47.26 ± 5.71	472.85 ± 23.64	200.12 ± 10.03
Roots	Water	54.06 ± 4.20	42.48 ± 3.77	641.07 ± 32.05	118.82 ± 9.11
	Dichloromethane	62.00 ± 5.20	77.05 ± 6.85	196.96 ± 9.84	52.45 ± 2.62
	Hexane	68.51 ± 6.17	124.94 ± 11.74	42.50 ± 2.12	11.22 ± 0.56
	Methanol	52.44 ± 3.32	45.26 ± 3.91	683.75 ± 34.18	176.22 ± 9.18

Values are expressed as mean ± standard deviation (SD) of three independent experiments (n = 3).

Phenolic Compound Profiles in Methanol Extracts of Plants

As part of this study, the phenolic composition of methanol extracts obtained from the above-ground parts of the *C. birecikensis* plant was analyzed qualitatively and quantitatively using the LC-MS/MS technique. Based on the chromatographic data obtained, the primary component identified in the methanol extract was fumaric acid (3398.75 µg/L). This primary component was followed by vanillic acid (2248.69 µg/L) and syringic acid (710.88 µg/L). Detailed phenolic profile distributions and quantitative values for the plant extracts in question are presented in Table 3.

Table 3. Phenolic contents of the *C. birecikensis* extract

Phenolic Compounds	Measure (µg/L)
Luteolin	390.35
Acetohydroxamic acid	116.80
Curcumin	4.08
Vanillic acid	2248.69
Naringenin	83.37
Syringic acid	710.88
Quercetin	86.78
Fumaric acid	3398.75
Ellagic acid	413.31
Caffeic acid	576.69
Oleuropein	18.51
Hydroxycinnamic	408.48
Salicylic acid	214.22
Hydroxybene	189.22

Additional Content Analysis of Plant Extracts

The levels of glucose, folic acid, and vitamin B12 in the methanol extracts were quantitatively determined using a chemiluminescence method. In the analyses conducted, glucose concentration in the aboveground parts of *C. birecikensis* was found to be 2.26 ng/mL, vitamin B12 0.048 ng/mL, and folic acid >24 ng/mL.

Upon examining the plant's basic elemental composition, it was observed that potassium (13,530 mg/kg) and calcium (10,820 mg/kg) were predominant in terms of mineral content; these elements were followed by sodium (1,293 mg/kg) and magnesium (591.3 mg/kg), respectively.

When the amino acid profile was analyzed, proline (4,600.45 µmol/L) and alanine (682.774 µmol/L) emerged as the most abundant components in the *C. birecikensis* methanol extract. In terms of essential amino acids, valine (434.149 µmol/L) has the highest concentration, followed by threonine, isoleucine, leucine, and phenylalanine. At lower levels, lysine (50.225 µmol/L), histidine (25.954 µmol/L), methionine (0.989 µmol/L), and tryptophan (0.168 µmol/L) were detected.

Furthermore, in the analysis of carnitine derivatives, free carnitine (2.65 µmol/L) and acetylcarnitine (1.26 µmol/L) were found to be the most prominent quantitative fractions in the extract.

Investigation of the Cytotoxic Activities of Plant Extracts

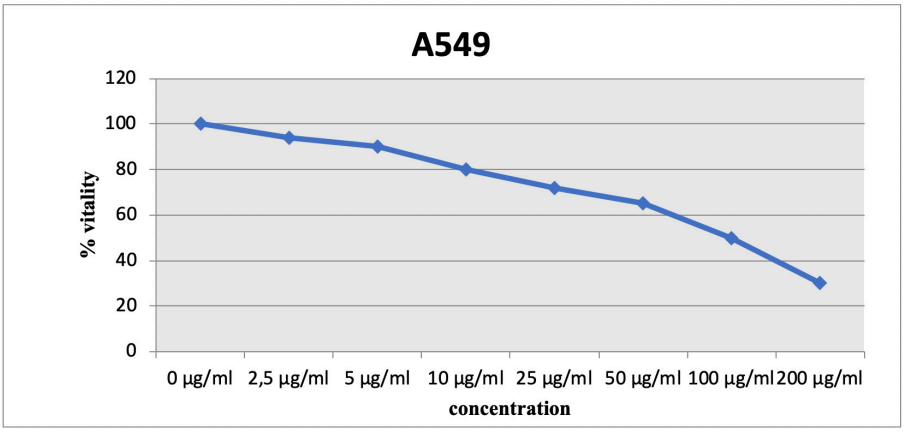
The cytotoxic potential of methanol extracts prepared from the aboveground parts of the *C. birecikensis* plant on cancerous and healthy cell lines was analyzed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and evaluated based on IC_{50} values.

When cell line-specific responses were examined, it was determined that *C. birecikensis* extracts exhibited significant antitumor activity against the A549 (lung adenocarcinoma) cell line. In comparative analyses with normal lung epithelial cells (BEAS-2B), it was determined that the extract's cytotoxic effect manifested at much higher doses in healthy cells compared to cancer cells.

As a result of the dose-response analyses conducted, it was determined that the inhibitory effect of the *C. birecikensis* extract on the proliferation of A549 lung cancer cells became evident at concentrations of 50 µg/mL and above.

This trend of decreased cell viability continued in parallel with increasing doses, and the half-maximal inhibitory concentration of the extract on the relevant cell line was calculated to be IC_{50} 89.67 µg/mL. This is shown in Figure 1.

Figure 1. Percentage viability graph of *C. birecikensis* extract on A549 cell line.



Investigation of the apoptotic effects of plant extracts using the Annexin-V method

The results of the Annexin-V analysis conducted on A549 cell lines quantitatively demonstrated the effect of *C. birecikensis* extract on programmed cell death. Under standard normoxic incubation conditions—37°C, 5% carbon dioxide, and 21% oxygen—the viability rate of untreated control group cells was determined to be 93.8%; the proportion of apoptotic and necrotic cells within the total population remained at 6.2%.

In contrast, in the experimental group treated with *C. birecikensis* extract at a concentration of 100 µg/mL, a significant decrease in cell viability was observed, dropping to 63.5%. At the same dose, the proportion of cells undergoing apoptotic and necrotic processes was found to rise to 36.6%. These data confirm that the applied extract significantly triggered cell death mechanisms (apoptosis/necrosis) in A549 lung cancer cells. This is shown in Figure 2 and Figure 3.

Figure 2. Annexin-V Result of A549 Cell Line

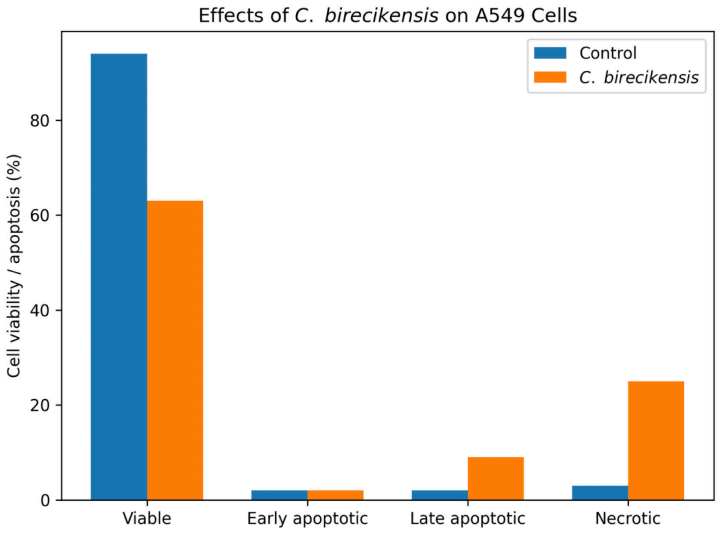
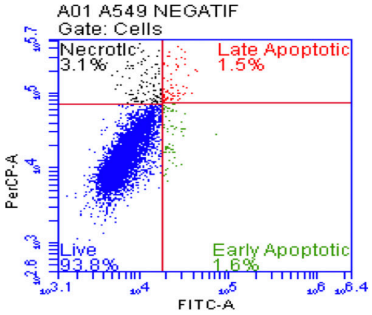
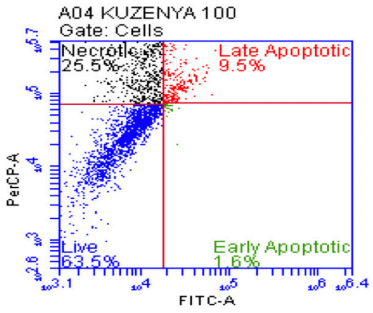


Figure 3. Demographic graph of Annexin-V Result of A549 Cell Line



Negative



C. birecikensis

DISCUSSION

The primary focus of this study is to identify the biological activities of the *C. birecikensis* plant with the aim of providing new natural resources for the treatment of cytotoxic activity and similar complex diseases.

In the extraction processes conducted on the flower, leaf, and root tissues of the *C. birecikensis* plant, it was observed that the phytochemical distribution varied depending on the polarity of the solvent. It was determined that the total phenolic content reached its highest values in methanol extracts in all three plant parts. When examining flavonoid distribution, methanol proved to be the most effective solvent in the flower and root sections, while water provided a more efficient extraction in the leaf tissue. A study conducted in the literature examining the Baccaurea angulata species supports our findings; as that study also reported that methanolic extracts possess the highest polyphenolic content and significant antiradical activity. This can be explained by methanol's selective success in extracting phenolic compounds and carotenoids, which exert an inhibitory effect on free radicals due to their ability to donate hydrogen (16).

A similar trend was also observed in a previous study. When the phytochemical profile of the Centaurea lycaonica species was examined, it was reported that methanol extracts exhibited high total phenolic and flavonoid concentrations, a finding that showed a strong correlation with the data from our study (17).

Based on radical scavenging activity (DPPH), the most pronounced activity values for all three morphological parts of the *C. birecikensis* taxon were recorded in the methanol extracts. Methanol has once again proven to be the most efficient solvent for the extraction of antioxidant components in flower, leaf, and root samples. Lower SC_{50} values observed in methanol extracts further support their strong antioxidant capacity, consistent with their higher phenolic content. The radical scavenging results we obtained for *C. birecikensis* are consistent with the data presented here. Researchers have determined that methanol and water fractions of the Centaurea lycaonica species exhibit significant antioxidant potential; this parallels the solvent efficacy observed in our study (17).

When examining the inhibitory effects of *C. birecikensis* samples on the ABTS radical, methanol extracts stood out in the flower parts, while aqueous extracts were observed to offer a more dominant antioxidant profile in the leaf and root sections. Lower SC_{50} values observed in methanol extracts further support their strong antioxidant capacity, consistent with their higher phenolic content. A study in the literature aligns with our findings that the activities of methanol and water solvents in ABTS analysis are comparable. Although methanol demonstrated slightly higher performance in the study in question, it was emphasized that both solvents were effective (18). Furthermore, the data obtained confirm the effectiveness of these two polar solvents in radical scavenging capacity, thereby reinforcing the validity of our study (17).

When the antioxidant potential of different morphological parts of *C. birecikensis* was examined using the FRAP method, it was observed that methanol was the most effective extraction solvent in flower and root samples, while water was the most effective in leaf samples. These data from our study are consistent with the results presented by Fatullayev et al. The researchers confirmed that the FRAP-based antioxidant levels in the methanol extracts of the plant species in question were superior to those of other solvent groups (17).

When the antioxidant potential of extracts obtained from the morphological parts of *C. birecikensis* was examined using the CUPRAC method, it was observed that methanol was the most effective solvent in all flower, leaf, and root samples. These results align with the data from the study conducted by Kubik et al. on taxa belonging to the Centaurea genus. The researchers emphasized that combinations of methanol and water also offer high reducing capacity, thereby confirming the solvent efficacy observed in our study (19).

An analysis of the compositional profile of the methanolic extracts revealed that *C. birecikensis* contains high levels of fumaric acid and vanillic acid. These findings are consistent with those of Boussoussa et al., who identified vanillic and syringic acids as the primary antioxidants in Rhanterium adpressum extracts (20). The roles of these phenolic compounds in vital processes—such as the regulation of endothelial function, the suppression of inflammation, and the balancing of blood pressure as an ACE inhibitor—have previously been reported by Liu et al. (21). Therefore, our study demonstrates that *C. birecikensis* is a strategic candidate as a botanical raw material for the production or supplementation of these biologically active molecules.

sis within the scope of the study revealed that *C. birecikensis* is source of potassium, calcium, and sodium. As emphasized by Baj et al. in the literature, the importance of calcium in bone development and neural regulation, the cardiovascular protective effect of potassium, and the vital role of sodium in electrolyte balance enhance the pharmaceutical value of this plant (22). The mineral profile data obtained from our study are consistent with the findings of Kovacik et al. (23).

Our research findings have revealed that the *C. birecikensis* taxon possesses an amino acid profile rich in proline and alanine. As emphasized by Raza and Linju in the literature, proline enhances the plant's resistance to environmental stressors through its ability to maintain cellular integrity and regulate oxidative stress pathways (24, 25). From a pharmacological perspective, it is essential for connective tissue health and collagen production. Similarly, alanine content plays a strategic role in regulating liver functions and the activity of aminotransferase enzymes through enzyme-substrate interactions in the body (26).

Carnitine, a key component of fatty acid metabolism in human physiology, plays critical roles in establishing hormonal balance and energy storage processes. This compound is primarily obtained exogenously through animal-based foods (meat, milk, and dairy products). Although animal sources are predominant, Jacques et al. have demonstrated the presence of carnitine in plant tissues and its biological significance through their studies on various plant species (27). In this study, the levels of free carnitine and acetyl carnitine detected in the *C. birecikensis* extract were recorded as the highest values among the measured parameters. The presence of free carnitine in the mitochondrial matrix enables the synthesis of acetyl-L-carnitine, which results in the reduction of acetyl-CoA, while also supporting the production of free CoA, which is essential for the continuity of the glycolytic pathway (28). Additionally, according to our analysis results, glucose, folic acid, and vitamin B12 were detected in the *C. birecikensis* extract. A deficiency in folic acid, which plays an active role in cellular growth, development, and amino acid metabolism cycles, can pave the way for serious pathologies such as anemia and carcinogenesis. Similarly, in cases of deficiency in components such as vitamin B12—which is of vital importance for maintaining the integrity of the nervous system and cell proliferation—replacement

through diet or dietary supplements is necessary (29).

As part of our studies, the cytotoxic potential of *C. birecikensis* extract on A549 and BEAS-2B cell lines was evaluated. The findings revealed that the extract exhibited significant cytotoxicity on both cell lines. Importantly, it was determined that the concentration required for the *C. birecikensis* extract to exert a cytotoxic effect on BEAS-2B cells was higher compared to that required for the A549 cell line. This indicates that the extract's inhibitory effect on the cancer cell line (A549) occurs at lower doses compared to BEAS-2B cells, which can be considered a control line.

According to the results of the Annexin-V analysis, the viability rate in the control group of A549 cells under normoxic conditions was 93.8%, while the total percentage of apoptotic and necrotic cells was recorded as 6.2%. In contrast, in the group treated with a 100 µg/mL dose of *C. birecikensis* extract, cell viability was found to have dropped to 63.5%, while the total proportion of apoptotic and necrotic cells rose to 36.6%.

All these findings demonstrate that the extracts possess biological cytotoxicity against lung cancer cell lines. Upon reviewing similar studies in the literature, it is observed that the results of the research conducted by Al Sharie et al. are consistent with our data. In the aforementioned study, it was reported that zinc oxide nanoparticles synthesized using the methanolic extract inhibited the proliferation of A549 human alveolar basal epithelial adenocarcinoma cells in a dose-dependent manner. MTT assays revealed that the methanolic extract exhibited preliminary cytotoxic activity against the A549 cell population (30). The results obtained in our study also support the therapeutic potential of plant extracts against lung cancer.

This study has several limitations. First, the cytotoxic activity was evaluated using only a single cancer cell line (A549), which limits the generalizability of the findings. Second, the molecular mechanisms underlying the observed cytotoxic effects were not investigated. Additionally, no in vivo validation was performed. Therefore, the present results should be considered as preliminary findings, and further studies involving multiple cancer models, mechanistic analyses, and in vivo experiments are required.

CONCLUSION

Within the scope of this study, the phytochemical profile, antioxidant capacity, and cytotoxic potential of plant extracts from *C. birecikensis*, an endemic species, were comprehensively analyzed.

Methanol and water extracts were found to exhibit high activity in DPPH, ABTS, FRAP, CUPRAC, TPC, and TFC assays. These data confirm that *C. birecikensis* possesses radical scavenging potential. In the conducted content analyses, fumaric acid and vanillic acid emerged as the primary components. Due to its rich phenolic and flavonoid content, it is anticipated that this plant could be utilized as a natural antioxidant source in the pharmaceutical or food industry by neutralizing free radicals. While the concentrations of potassium (K), calcium (Ca), and sodium (Na) in the plant are measured at the highest levels, the absence of heavy metals with toxic properties is critical for the plant's safety profile.

Proline and alanine were identified as the predominant components in the amino acid profile. Additionally, the detection of high levels of free carnitine and acetyl carnitine in the extracts indicates the plant's importance in metabolic processes. The presence of glucose, as well as vital micronutrients such as folic acid and vitamin B12, in the extracts enhances the plant's nutraceutical value.

It has been found that *C. birecikensis* extract exhibits a preliminary cytotoxic effect on lung cancer cell lines. The biomolecular richness of this endemic species highlights its potential for use in disease prevention or as a supportive therapy. Additionally, it possesses the potential to serve as a value-added commercial product for the regional economy.

In conclusion, *C. birecikensis* exhibits notable antioxidant capacity and preliminary in vitro

cytotoxic activity against A549 lung cancer cells. However, these findings are limited to in vitro conditions and a single cell model. Further studies, including mechanistic investigations and in vivo validation, are necessary to clarify its potential biological and pharmacological relevance.

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