https://doi.org/10.31925/farmacia.2023.6.11 **ORIGINAL ARTICLE**

INVESTIGATION OF ANTI-INFLAMMATORY, ANTIBIOFILM, ANTIOXIDANT AND CYTOTOXIC ACTIVITIES OF *CYCLAMEN HEDERIFOLIUM* (*PRIMULACEAE*)

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Abstract

Cyclamen L. is a tuberous geophyte, and some species of this genus have been used for their biological activities in folk medicine. However, studies on the tuber of *Cyclamen hederifolium*, which is known to contain saponins, are limited. In the present study, some biological activities of *C. hederifolium* tuber extract were investigated, along with its total saponin content. The antioxidant activity of the extract was performed by DPPH radical scavenging and metal chelating methods. To test the anti-inflammatory activity of the extract, LPS-stimulated RAW 264.7 macrophage cells were treated in subsequent experiments, and the levels of pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12) and nitric oxide (NO) were measured by ELISA and Griess assay, respectively. Also, the antibacterial and antibiofilm activities of the extract were evaluated. While the minimum inhibitory concentrations (MICs) of the extract were determined to be 1.56 mg/mL for both *S. aureus* and *E. faecalis*, the highest percentage biofilm inhibition of the extract was detected on *E. faecalis* at 80.70%. The extract potently scavenged DDPH radical (IC_{50} 4.22 µg/mL) and showed the ability of metal chelating (24.12 mg EDTAEs/g). According to the anti-inflammatory activity tests, *C. hederifolium* inhibited not only NO production, but also the levels of proinflammatory cytokines. As for the cytotoxic activity, the extract caused a decline in SH-SY5Y (34.03%) and HeLa (72.18%) cancer cell viability compared to the control cells. The results suggest that the tuber extract of *C. hederifolium* has some biological properties that may be due to the presence of saponins for pharmaceutical applications.

Rezumat

Cyclamen L. este un geofit tuberos, iar unele specii din acest gen au fost folosite pentru activitățile lor biologice în medicina tradițională. Cu toate acestea, studiile asupra tuberculului de *Cyclamen hederifolium*, despre care se știe că acesta conține saponine, sunt limitate. Astfel, au fost investigate unele activități biologice ale extractului de tubercul de *C. hederifolium*: activitatea antioxidantă a extractului a fost studiată prin metode de captare a radicalilor DPPH și de chelatare a metalelor; activitatea antiinflamatoare a extractului a fost evaluată folosind celule macrofage RAW 264.7 stimulate cu LPS și determinând nivelurile de citokine proinflamatorii (TNF-α, IL-1β, IL-6 și IL-12) și oxid nitric (NO) prin metoda ELISA și, respectiv, prin testul Griess. De asemenea, au fost evaluate activitățile antibacteriene și antibiofilm ale extractului. În timp ce concentrațiile minime inhibitorii (MIC) ale extractului au fost determinate ca 1,56 mg/mL atât pentru *S. aureus*, cât și pentru *E. faecalis*, cel mai mare procent de inhibare a biofilmului a fost detectat folosind *E. faecalis* (80,70%). Extractul a chelatat radicalul DDPH (IC_{50} 4,22 µg/mL) și metalele (24,12 mg EDTAE/g). Conform testelor de activitate antiinflamatoare, *C. hederifolium* a inhibat nu numai producția de NO, ci și nivelurile de citokine proinflamatorii. În ceea ce privește activitatea citotoxică, extractul a provocat o scădere a viabilității celulelor canceroase SH-SY5Y (34,03%) și HeLa (72,18%) în comparație cu martorul. Rezultatele sugerează că extractul de tubercul de *C. hederifolium* prezintă proprietăți biologice care se pot datora prezenței saponinelor pentru aplicații farmaceutice.

Keywords: antibiofilm, antioxidant, *Cyclamen*, cytotoxic, macrophages

Introduction

Cyclamen genus (*Primulaceae* family), comprising 23 species, is primarily distributed across the Mediterranean region. The ethnobotanical evidence has claimed the use of species from *Cyclamen* L. in traditional medicine [1]. Previous studies have reported the use of *Cyclamen* species to treat a number of problems, including dermal infections, dental problems, wounds, haemorrhoids, diabetes, arthritis, ear infections, parasites, female/male infertility, gastrointestinal problems, respiratory issues, vaginal and cervical problems, genito-urinary diseases, dysmenorrhea, and is also used as sedative, laxative, contraceptive and abortive [2, 3]. *Cyclamen hederifolium* Aiton is a plant with a common Mediterranean element in Turkey, known as ivy-leaved cyclamen. Its tubers are pounded and placed in a certain amount of water, and then the filtered water is used as a pesticide to kill harmful insects on

the tobacco plantation. This effect is due to the fact that the saponins in the tubers of *C. hederifolium* [4].

Antibiotics are significant drugs used for the treatment of infections in humans and animals. Due to the consistent increase in the resistance of microbes to antimicrobials and the decreasing ability of the available antimicrobials to treat common infections, antimicrobial resistance (AMR) is becoming a more serious health threat worldwide [5, 6]. Considering the time-consuming, comprehensive and expensive drug development process from discovery to clinical use of a new antimicrobial molecule, recent studies have focused on potential antimicrobial molecules obtained from different natural sources. Plant extracts may be rich reservoirs of various compounds that have antimicrobial activity, and thus they may be beneficial for fighting antimicrobial resistance [7, 8].

In addition to intrinsic or acquired antibiotic resistance mechanisms, biofilm formation is one of the major virulence factors and antimicrobial resistance strategies of bacteria. It is known that the bacteria in the biofilm layer that may occur on biotic/abiotic surfaces may be 100 - 1000 times more resistant to antibiotics and disinfectants [9]. Therefore, it is critical to investigate the antibiofilm activities of new molecules, in addition to their antimicrobial activity profiles, while developing novel antimicrobial treatment strategies.

Free radicals are generated by various metabolic processes, and the uncontrolled or increased formation of free radicals in the body may lead to oxidative stress. The balance between free radicals and anti-oxidants is important for health [10]. Oxidative stress plays an essential role in the pathophysiology of various diseases like cancer, chronic inflammation, cardio-vascular and neurodegenerative diseases. Although the oxygen-free radical hypothesis has been known for over 50 years, the role of oxidative stress in the progression of diseases has been identified in the last two decades. Afterwards, studies on antioxidants for preventing and treating these diseases gained significant importance [11, 12].

Growing scientific evidence suggests that chronic inflammation may lead to serious diseases, such as heart disorders, obesity, diabetes and cancer. Therefore, natural products have attracted more attention due to their anti-inflammatory properties [13]. Macrophages are essential for host immunity, and by detecting pathogenic substances, they initiate and regulate inflammatory responses [14]. Natural plant compounds that can suppress the production of inflammatory mediators in activated macrophages can act as potential anti-inflammatory agents.

From our literature search, there is a paucity of scientific information regarding the bioactivity of *C. hederifolium*. In this regard, the current work endeavours to bring the possible anti-inflammatory, antibacterial, antibiofilm, antioxidant and cytotoxic activities of the ethanolic extracts of *C. hederifolium* tuber parts into the limelight of the scientific community.

Materials and Methods

Plant material and extraction

C. hederifolium was collected in 2016 from Manisa, Turkey, during the hibernate stage and identified (Voucher No.: C. Ozay 3011) by Asst. Prof. Cennet Ozay, Izmir Katip Celebi University, Turkey. The tubers of the plant were air-dried over shadow, and powdered to a fine grain. The extract was prepared using ethanol in a shaker water bath for 12 hours. Subsequently, the extract was concentrated at a reduced temperature and pressure and then lyophilised. The crude extract was kept at -20°C until needed [15]. The dried extract was weighed to determine the percent yield. The percentage yield was obtained using this formula:

$$W_1/W_0 \times 100,$$

where W_1 is the final weight of the extract and W_0 is the initial weight of the sample.

Total saponin content

The total saponin content was determined by the vanillin-sulfuric acid method [16]. The extract was mixed with the same amount of vanillin (8%, w/v) and twice the amount of sulfuric acid (72%, w/v). The mixture was incubated at 60°C for 10 minutes followed by cooling in an ice water bath for 15 minutes. Absorbance was measured at 535 nm. The total saponin content was expressed as equivalents of Quillaja (mg QAEs/g).

Antioxidant activity

DPPH radical-scavenging antioxidant activity

The capacity of the extract to eliminate the 1,1-di-phenyl-2-picrylhydrazyl (DPPH) radical was evaluated using the method of Blois [17]. The reaction consisted of 40 µL of the extract at different concentrations and 160 µL of a methanolic solution of DPPH radical in a 96-well microplate. The mixture was allowed to incubate for 30 min in the dark at room temperature. Absorbance was measured at 517 nm using a micro-plate reader. The synthetic antioxidant BHT (butylated hydroxytoluene) was used as a positive control. The results were indicated as IC_{50}.

The ability of the extracts to scavenge DPPH was expressed in percent inhibition calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100,$$

where A_0 is the absorbance of the control and A_1 is the absorbance of the extract at 30 minutes.

Metal chelating activity

The ferrous chelating capacity of the extract was determined with Dinis *et al.*'s [18] method with slight modifications. According to this method, the extract inhibits ferrozine complexing with Fe^{2+}, and colour expansion is determined spectrophotometrically (562 nm).

The results were provided as ethylenediaminetetraacetic acid (EDTA) equivalents (mg EDTAEs/g extract).

Anti-inflammatory activity

Cell viability assay

The extract used in all cell culture assays was diluted in the growth media of the RAW 264.7 cell line. The vehicle for the initial stock of the extract was 0.1% dimethyl sulfoxide (DMSO). The effect of *C. hederifolium* on cell viability was determined by the MTT assay. RAW 264.7 cells were mechanically scraped and plated at 5×10^3 cells/well in 96-well plates containing 100 µL of DMEM medium with 10% heat-inactivated FBS and incubated for 24 hours (37°C and 5% CO_2). After incubation, the cells were treated with different concentrations of the extract (25 - 200 µg/mL) for 24 hours. Then, MTT solution was added to the wells and incubated for four hours at 37°C. Finally, DMSO was added to dissolve the formazan crystals, and absorbance was measured at 570 nm. Cell viability (%) was calculated as follows:

$$\text{Cell viability (\%)} = (Abs_{sample}/Abs_{control}) \times 100.$$

Griess nitrite assay

The NO production was measured as described by Han *et al*. [19]. RAW 264.7 cells at 5×10^3 cells/well were cultured in 96-well plates in triplicate for 24 hours and pre-incubated with different concentrations of the extract (12.5 - 50 µg/mL) for one hour and were then stimulated with LPS (1 µg/mL). The culture supernatants were collected 24 hours after the LPS stimulation, and the concentrations of NO were measured according to the Griess reaction. 50 µL of supernatant was mixed with an equal volume of Griess reagent (1% sulfanilamide and 0.1% naphthyl-ethylenediamine in 5% phosphoric acid) and incubated for 10 min at room temperature, then the absorbance was measured at 550 nm. Finally, the concentration of nitrite was calculated from a standard curve drawn with known concentrations of sodium nitrite dissolved in DMEM.

Pro-inflammatory cytokine determination

RAW 264.7 cells were pretreated with different concentrations of the extract (12.5 - 50 µg/mL) for one hour, and then LPS (1 µg/mL) was added to the treatment cells to activate the macrophages. After 24 hours of incubation of cells, the supernatants of cell cultures were collected and used for measuring the levels of TNF-α, IL-1β, IL-6 and IL-12 proteins using an ELISA kit (Boster, USA) according to the manufacturer's instructions. ELISA results were recorded using a microplate spectrophotometer at 450 nm, and the corrected absorbance was noted as *per* the manufacturer's instructions. Results were expressed in pg/mL. Each sample was measured in triplicate, and values were derived from the standard curve.

Antibacterial activity

Test microorganisms and media

Antibacterial activity profiles of the *C. hederifolium* extract against gram-negative (*E. faecalis*) and gram-positive (*S. aureus*) bacterial strains were investigated by disk diffusion test and broth microdilution method (BMD) in accordance with the recommendations of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) in this study [20]. The anti-microbial susceptibility tests were carried out using the following American Type Culture Collection (ATCC) bacterial strains: *Staphylococcus aureus* ATCC 29213, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853. All the bacterial strains were stored at -80°C in Brain-Heart Infusion Broth (BHIB, Merck) that contains 10% glycerol until use. Before performing the antimicrobial activity tests, the bacterial strains were grown on Mueller-Hinton Agar (MHA) with an overnight incubation at 37°C. *Enterococcus faecalis* was used as a positive control strain for biofilm quantification experiments. Mueller-Hinton Broth (MHB) and Tryptic Soy Broth (TSB, supplemented with 2% glucose) were used for BMD and biofilm formation experiments, respectively.

Disk diffusion method

Kirby-Bauer disk diffusion susceptibility test was performed to detect the antibacterial activity of *C. hederifolium* extract. Bacterial suspensions were prepared from fresh bacterial colonies on MHA by using sterile physiological saline. The microorganism suspensions were adjusted to 0.5 McFarland turbidity (approximately 1 - 2 x 10^8 CFU/mL) by a densitometer device (Biosan, DEN-1). The suspensions were spread to the agar plates by using sterile cotton swabs. Following that, 10 µL of the extract (100 mg/mL) was absorbed into sterile blank discs (6 mm, Oxoid), and the discs were placed on the agar plates. The plates were incubated at 37°C for 24 hours. Ciprofloxacin discs were used for internal quality control, and ethanol was also examined alone. The inhibition zone diameters (mm) on agar plates were measured for each strain at the end of the incubation period. All the experiments were performed in triplicate.

Broth microdilution method

The minimum inhibitory concentrations (MICs) of *C. hederifolium* extract against bacterial strains were determined by BMD [20]. Initially, the bacterial strains were grown on MHA at 37°C overnight. Fresh colonies of each strain were suspended in sterile physiological saline. The bacterial suspensions were adjusted to the 0.5 McFarland turbidity standard. These suspensions were diluted in a ratio of 1/100 to get the final inoculum concentration (5 x 10^5 CFU/mL). Initially, 50 µL of MHB were added to the first wells of sterile, 96-well U-bottom microplates. Then appropriate concentrations of *C. hederifolium* extract (50 µL) were added to the first wells, and serial dilutions were prepared using the side wells of the microplates. Lastly, the bacterial suspensions (50 µL) were inoculated into each well. The microplates were incubated at

37°C for 24 hours for bacterial growth. The lowest extract concentration that inhibited bacterial growth was determined as the MIC value of the extract. All the experiments were performed in triplicate.

Antibiofilm activity

Crystal violet (CV) staining method

The antibiofilm effect of the *C. hederifolium* extract was quantified using a modified spectrophotometric microplate method using crystal violet (CV) [21, 22]. Initially, bacterial strains were grown on MHA at 37°C overnight. After the incubation period, the bacterial suspensions with turbidity adjusted to a 0.5 McFarland turbidity standard were prepared in sterile tubes containing TSBG (3 mL) by the direct colony suspension method. Mixtures of 180 µL of TSBG and 20 µL of the bacterial suspension were added to the wells of sterile, 96-well flat-bottom microplates. Medium control wells containing only 200 µL of TSBG were used in each microplate. The microplates were incubated at 37°C for 24 hours. Following the incubation, the wells were aspirated and washed three times with sterile phosphate buffered saline (200 µL) (Oxoid, UK) in order to remove non-adherent cells. Then the microplate wells were allowed to dry at room temperature. The remaining attached microorganisms were fixed by adding 200 µL of methanol for 15 minutes. Following that, 0.1% CV solution (200 µL) was added to each well. After 15 minutes, the wells were aspirated and washed under tap water until the rinse water became colourless. Lastly, the microplates were dried at room temperature, and each well was destained with 200 µL of 95% ethanol for 15 minutes. The spectrophotometric measurements were performed at 570 nm using a microplate reader (Varioskan Flash, Thermo Fisher Sci., Inc., USA). The optical density (OD.) values of the wells that contained only TSBG medium were used as negative controls. *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 were used for biofilm production. The cut-off OD. (OD.c) was defined as three standard deviations above the mean OD. of negative controls. All tests were carried out in triplicate. Biofilm production capacities for bacterial isolates were categorised according to the following criteria: OD. ≤ OD.c: no biofilm production; OD.c < OD. ≤ (2 × OD.c): weak biofilm producer; (2 × OD.c) < OD. ≤ (4 × OD.c): moderate biofilm producer; and (4 × OD.c) < OD.: strong biofilm producer. The percentages of the biofilm inhibition (%) were determined according to the formula given below:

$$\text{Percentage Biofilm Inhibition (\%)} = (OD._A - OD._B)/ O.D._A \times 100,$$

where, $OD._A$: The optical density of biofilm controlled well and $OD._B$: The optical density in the presence of the plant extract.

Cell culture and cytotoxicity assay

The potential cytotoxic activity of *C. hederifolium* tuber extracts on human neuroblastoma (SH-SY5Y, ATCC #CRL-2266) and human cervical carcinoma (Hela, ATCC #CCL-2) cell lines was determined by colorimetric 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) reagent as previously described in detail [23]. Cell lines were obtained from American Type Culture Collection (ATCC, USA) and maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum and two mM L-glutamine supplemented at 37°C in a humidified atmosphere of 5% CO_2 air.

Cells were seeded into 96-well plates at a density of 7500 cells/well, and plates were incubated overnight to perform cytotoxicity experiments. Subsequently, cells were treated with the extracts (100 µg/mL) for 24 hours. Then, cells were incubated with 200 µL of DMEM containing 5 mg/mL MTT reagent for four hours. Eventually, DMEM was taken out and the formazan crystals were resolved by 100 µL of DMSO. The microplate reader measured the absorbance of wells at 570 and 650 nm. Each experiment was done in triplicate. The cell viability was calculated as a percentage (%) of DMSO-treated control cells. Results were represented as the mean ± standard deviation (Mean ± SD) of three independent assays.

Statistical analysis

The results obtained in this study are expressed as the mean ± standard deviation (SD). Statistical analysis and data processing were performed using SPSS 22.0. Comparisons of the treatments among groups were analysed by one-way ANOVA with a post-hoc Tukey's test. The significance was accepted as $p \leq 0.05$.

Results and Discussion

The yield of the extract, total saponin content and antioxidant activity

The percentage yield of *C. hederifolium* tubers was recorded at 33.11% (Table I). The total saponin content of the extract was calculated as equivalent to Quillaja and found to be 123.35 ± 2.06 mg QAEs/g.

Table I

Extract yield, total saponin content and antioxidant activity of *C. hederifolium* (mean ± SD)

	***C. hederifolium* tuber extract**
Extraction yield (%)	33.11 ± 0.25
TSC (mg QAEs/g)	123.35 ± 2.06
DPPH assay (IC_{50} value, µg/mL)	4.22 ± 0.01
Metal chelating activity (mg EDTAEs/g)	24.12 ± 0.18

TSC: total saponin content; *QAEs:* Quillaja equivalents, *EDTAEs:* EDTA equivalents.

The antioxidant activity of the extract is shown in Table I. While the DPPH radical scavenging activity of *C. hederifolium* was determined to have an IC_{50} value of 4.22 ± 0.01 µg/mL, the metal chelating capacity of the extract was determined to be 24.12 mg EDTAEs/g.

Anti-inflammatory activity

The effect of *C. hederifolium* on RAW 264.7 macrophage cell viability is shown in Figure 1. The RAW 264.7 cell viability assay was the preliminary study to test the effect of *C. hederifolium* tuber extract on RAW 264.7 cell viability. The aim of this assay is to determine the safe and non-toxic concentration for the next assay. Viability was measured by an MTT assay based on the conversion of yellow tetrazolium salt to form a purple formazan product. The percent cell viability was determined by comparing the cell viability value of the treatments to the control. This viability assay (Figure 1) shows that *C. hederifolium* tuber extract in given concentrations is still usable for the normal RAW 264.7 cells, except for the concentrations of 75, 100 and 200 µg/mL. At the highest concentrations of the sample, the RAW 264.7 cell viability was low, which indicates that these concentrations are a little bit toxic to the cells, so those concentrations will not be used in the next step of the assay. The NO production was measured in the RAW 264.7 cell culture supernatants. The amount of nitrite (µM), a stable metabolite of NO, significantly decreased due to the rising extract concentration (Figure 2). A 50 µg/mL *C. hederifolium* ethanol extract demonstrated the highest nitrite inhibitory activity. As for the pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12) levels, *C. hederifolium* has a greater effect on decreasing TNF-α, IL-6 and IL-12 levels than the IL-1β level.

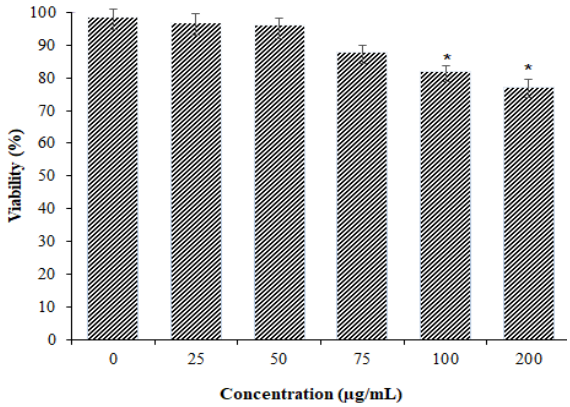

Figure 1.
The effect of C. *hederifolium* on cell viability of RAW 264.7 macrophage cells

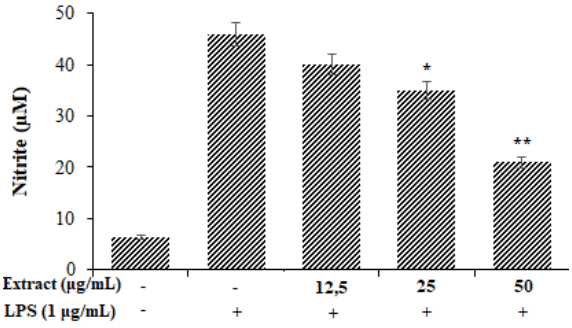

Figure 2.
The effect of C. *hederifolium* on NO production in LPS stimulated RAW 264.7 macrophages

As shown in Figure 3, there was a 4-fold and 3-fold reduction in LPS-induced IL-12 and IL-6 production, respectively, in RAW 264.7 cell was pre-treated with 50 µg/mL of extract.

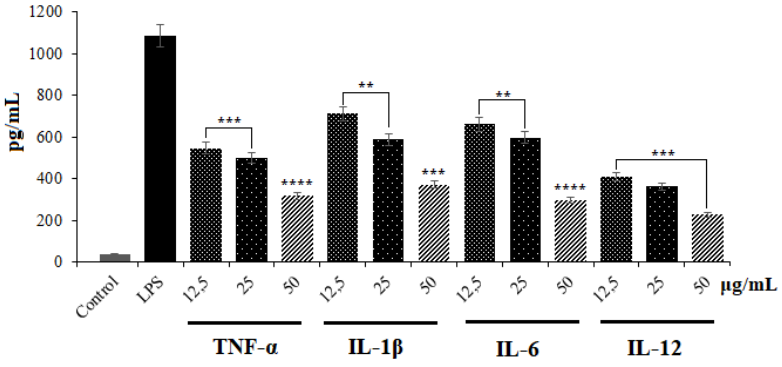

Figure 3.
The effect of *C.hederifolium* on pro-inflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12) production in LPS-stimulated RAW 264.7 macrophages

Antibacterial activity

According to disk diffusion test results, the inhibitory zone diameters of *C. hederifolium extract* against *S. aureus* and *E. faecalis* were determined to be 12 mm and 10 mm, respectively. No inhibition zone diameter was detected for other gram-negative strains (*E. coli* and *P. aeruginosa*). The MIC values of the *C. hederifolium* extracts against gram-positive two bacterial strains were investigated by the microdilution method,

and MIC results were detected as 1.56 mg/mL for *S. aureus* and *E. faecalis* (Table II).

Antibiofilm efficacy

Besides the antimicrobial activity, the antibiofilm effect of the extract on bacterial biofilm formation was also investigated in the present study. The biofilm-forming capacities of two bacterial strains and the effects of the extract on their mature biofilm were investigated by the CV method based on spectrophotometric measurement. The optical density values measured at 570 nm, the biofilm-forming capacity of the strains and the percentage of biofilm inhibition in the presence of *C. hederifolium* extract are shown in Table II.

Table II

The minimum inhibitory concentrations (MICs) and antibiofilm effects of *C. hederifolium*

Bacterial Strain	MIC (mg/mL)	OD. (570 nm)	Biofilm forming capacity	OD. in the presence of the extract	Percentage Biofilm Inhibition (%)
***S. aureus* ATCC 29213**	1.56	0.2443	Weak biofilm producer	0.1364	44.16↓
***E. faecalis* ATCC 29212**	1.56	0.4364	Strong biofilm producer	0.0840	80.70↓

MIC: Minimum Inhibitory Concentration, OD.: optical density.

Cytotoxic activity

The cytotoxic effects of the tuber parts of *C. hederifolium* ethanol extract were tested against human cancerous SH-SY5Y and HeLa cell lines. For this aim, cells were treated with extracts at a 100 µg/mL concentration. The cytotoxic activity was determined by the MTT method. Revealed data showed that *C. hederifolium* caused a significant decrease in SH-SY5Y and HeLa cell viability (Figure 4) compared to the control cells (34.03% ± 1.11 and 72.18% ± 4.0, respectively) ($p \leq 0.05$ and $p \leq 0.001$).

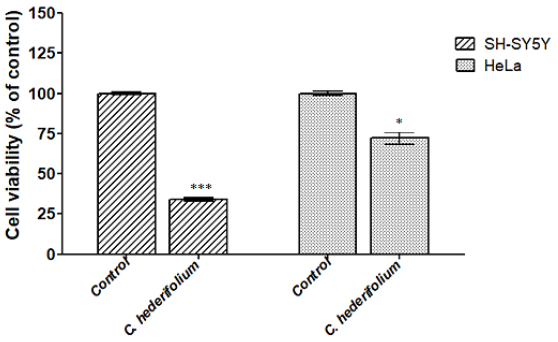

Figure 4.
Cytotoxic effects of *C. hederifolium* on SH-SY5Y and HeLa cell lines
Data are presented as mean ± SD

In the last three decades, huge progress in medicinal plant research has been observed. In fact, the global interest in the use of herbal remedies has created a huge need for information about the uses and therapeutic properties of these plants [24]. Turkey is home to a large diversity of plants. Floristic studies have shown that Turkey houses about 12,000 plant taxa on its soils, and more than 3000 of them are endemic. Geophytes are an important part of this rich biodiversity and contain many important endemic and endangered species [25].

As geophytes, the antibacterial activity of *C. cilicium*, *C. pseudibericum* and *C. hederifolium* tuber extracts has been reported from Turkey against four bacterial fish pathogens, namely, *Vagococcus salmoninarum*, *Staphylococcus epidermidis*, *Lactococcus garvieae* and *Yersinia ruckeri*. All the tested tuber extracts were detected to have moderate antibacterial activity [26]. Mansour *et al*. [27] reported that the methanolic extracts of *C. hederifolium* tubers have shown *in vitro* antimicrobial activities against *S. aureus* and *E. coli*, which are known to be common causative bacteria responsible for several infectious diseases. Conversely, it was indicated that the antimicrobial activity of both ethanol and aqueous extracts of *C. hederifolium* against 4 bacteria (*Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 6538, *Escherichia coli* ATCC 8739 and *Salmonella abony* NCTC 6017) and one fungal strain (*Candida albicans* ATCC 10231) was negative in another study [28]. Similarly, no inhibition zone was observed for gram-negative bacteria in our study. However, the extract showed an antibacterial effect against the two tested gram-positive bacteria (*S. aureus* and *E. faecalis).* Additionally, the antibiofilm activity of *C. hederifolium* tuber extract was also investigated, and high percentages of biofilm inhibition were detected on *S. aureus* and *E. faecalis.*

The plant secondary metabolites (PSMs) can be classified as phenols, terpenoids, alkaloids, glicosides and saponins [29]. Saponins are a class of PSMs that have been accepted as the most characteristic and important chemical constituents in *Cyclamen* species. The tubers of *Cyclamen* species are particularly rich in triterpene saponins [30, 31]. Phytochemicals/PSMs have the potential to act as antioxidants, releasing free radicals in response to oxidative stress. The controlled formation of free radicals and antioxidants protects cellular components from oxidative damage caused by ROS-containing chemical reactions [12]. These ions and radicals can initiate chain reactions that produce free radicals that cause oxidative damage to DNA, proteins and lipids. This causes cellular function loss and free radical-related diseases like atherosclerosis, neuronal degeneration, ischaemia-reperfusion damage and cancer [32]. In the present study, the tuber of *C. hederifolium* was analysed for its free radical scavenging activity by the DPPH method. The extract was potently scavenged by the

DDPH radical (IC_{50} 4.22 μg/mL). In another study, *Cyclamen africanum* demonstrated a perfect capacity for donating electrons and hydrogen by DPPH assay [33]. The metal-chelating ability of *C. hederifolium* tuber extract was also investigated. With regard to the role of transition metal in Fenton reactions, promoting the formation of radicals, and enhancing oxidative stress, metal-chelating agents represent therapeutic candidates. In the present study, *C. hederifolium* tuber extract exhibited metal chelating activity (24.12 mg EDTAEs/g). The metal chelating ability of *Cyclamen cilicium* tuber extract was reported earlier to be 21.67 mg EDTAEs/g extract [3].

It has been understood that the antimicrobial/antioxidant efficacy profiles of plants can vary depending on many factors, such as the growing environment, plant species and extraction method [34]. *Cyclamen* species are one of the medicinal plants that stand out with its potential antimicrobial/antioxidant effects [35]. While there are many studies on *C. mirabile* and *C. persicum* species in the literature, more limited studies have been reported on *C. hederifolium* species. With the findings of previous studies, *Cyclamen* spp. has been reported to exhibit *in vitro* antioxidant, antimicrobial and antifungal activities [30, 36, 37].

Saponins are steroid or triterpenoid glycosides common in a large number of plants, and several biological effects have been ascribed to saponins. Extensive research has been carried out into the membrane-permeabilising, immunostimulant, hypocholesterolaemic and anticarcinogenic properties [38]. Determining the *in vitro* cytotoxicity of plant extracts is the first step towards investigating the anticancer effects of compounds derived from natural sources. With this in mind, the cytotoxic effects of ethanol extract from the tuber parts of *C. hederifolium* were investigated on two cancerous (SH-SY5Y and HeLa) cell lines by the MTT method. *C. hederifolium* extract (100 μg/mL) caused a significant decrease in the tested cells viability compared to the control cells. When we look at the other studies in this field, the cytostatic effects and cell growth inhibitory activities of the ethanol tuber extract of *C. hederifolium* collected from Serbia against 4T1, HCT116, CT26 and LLC1 cancer cell lines were demonstrated by the MTT assay. It has been reported to exhibit potent cytotoxic activity against cell lines [28]. This cytotoxicity may be attributed to the saponins contained in the plant.

Since ancient times, a great variety of plants have been used for therapeutic purposes. Most parts of plants have been used as extracts and may possess anti-inflammatory properties related to diseases such as atherosclerosis, neurodegenerative diseases, or cancer [39]. Natural plant constituents that are able to suppress the production of inflammatory mediators in activated macrophages can act as potential anti-inflammatory agents. Lipopolysaccharide (LPS), an endotoxin derived from gram-negative bacteria, is a powerful activator of macrophage cells, and activated macrophages are known to produce inflammatory mediators [40]. The potential anti-inflammatory effects of *C. hederifolium* were examined, and the RAW 264.7 murine macrophage cell line, which is frequently used as an *in vitro* model in studies of inflammation, was selected in this study. We found that the extract inhibited both NO production and the levels of proinflammatory cytokines (TNF-α, IL-1β, IL-6 and IL-12). This effect may be substantiated due to inhibition of any mediators by existing phytochemicals such as glycosides or steroids present in the plant extract. A previous study showed that isolated a triterpene glycoside (repandoside) and two saponins (deglucocyclamin and anagalloside B) at 100 μM inhibited the LPS-induced IL-8 and TNF-α expressions in the human macrophage cell line THP-1 [41].

Conclusions

Medicinal plants, including several bioactive compounds known as plant secondary metabolites, have frequently been used worldwide for the treatment of various diseases. In particular, intensive research on the biological activity profiles of these medicinal plants and their components, which are responsible for these effects, is continuing. However, studies on the tuber of *Cyclamen hederifolium*, which is known to be a medicinal plant and contains saponins, are limited. This study was focused on examining the anti-inflammatory, antibacterial, antibiofilm, antioxidant and cytotoxic properties of *C. hederifolium*. The results suggest that tuber extracts of *C. hederifolium* have some biological properties, which may be due to the presence of saponins. To the best of our knowledge, the antibiofilm efficacy and anti-inflammatory effects of this plant were first studied and reported in this research. Further detailed phytochemical studies are needed to isolate and identify active saponin constituents.

Conflict of interest

The authors declared no conflict of interest in the manuscript.

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