ORIGINAL ARTICLE
(CC BY-SA)

UDC: 581.6::615.451.2
DOI: https://doi.org/10.2298/VSP190703096K

Chemical and pharmacological characterization of aqueous and ethanolic extracts of *Cyclamen hederifolium* Ait. (Primulaceae) tuber

Hemijska i farmakološka karakterizacija vodenog i etanolnog ekstrakta lukovica *Cyclamen hederifolium* Ait. (Primulaceae)

Ksenija Kojičić*, Aleksandar Arsenijević†, Marija Marković‡, Vesna Stankov Jovanović§, Zoran Simić||, Vanja Tadić¶, Snežana Cupara*

University of Kragujevac, Faculty of Medical Sciences, *Department of Pharmacy, †Department of Microbiology and Immunology, Faculty of Science, ||Institute of Chemistry, Kragujevac, Serbia; University of Niš, Faculty of Science, ‡Department of Biology, §Institute of Chemistry, Niš, Serbia; ¶Institute for Medical Plant Research "Dr. Josif Pančić", Belgrade, Serbia

Abstract

Background/Aim. *Cyclamen hederifolium* (*C. hederifolium*) Ait. belongs to the family Primulaceae, which includes 23 species of cyclamen, naturally distributed in the Central and Southern Europe, Western Asia and some parts of North Africa. This plant is considered highly poisonous and not suitable for human use. However, tuber extracts have been used in traditional medicine and homeopathy. The aim of this study was to investigate *C. hederifolium* growing naturally in Serbia for its metal content and biological activities (antioxidant, antibacterial, antifungal and cytotoxic activity). **Methods.** Content of metals was determined by atomic absorption spectrophotometric method. We used several different assays for assessment of antioxidant activity of both aqueous and ethanol extracts of *C. hederifolium:* 2.2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay, 2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay, ferric-reducing antioxidant power (FRAP) assay, total reducing power assay (TRP) and cupric reducing antioxidant capacity (CUPRAC) assay. The disk diffusion assay was used to investigate sensitivity of laboratory bacterial and fungal control strains against investigated extracts. Aqueous and ethanol extracts of *C. hederifolium* were examined on 4 different tumoral cell lines by *in vitro* MTT bioassay. **Results.** The presence of Mn, Ca, Mg, Fe, Zn, K, Cu was confirmed in aqueous and ethanol extract, as well as in whole tubers and soil, while Cr, Ni, Pb and Cd were not detected. Both aqueous and ethanol extract of *C. hederifolium* tubers showed antioxidant activity, that positively correlated to content of phenols and flavonoids in it. Aqueous extract was slightly superior in these terms than ethanol one. None of the tested extracts showed antimicrobial activity. Both investigated extracts showed cytotoxicity against four cancer cell lines 4T1, HCT116, CT26, LLC1, in the concentration range 15,625–2,000 μg/mL. Ethanol extract showed stronger cytotoxicity than aqueous extract. **Conclusion.** Seven metals were identified in the *C. hederifolium* tubers, extracts and soil. Both extracts exhibited antioxidant and cytotoxic activity.

Key words:
antioxidants; cyclamen; flower essences; metals; phytotherapy; spectrophotometry, atomic.

Apstrakt

Uvod/Cilj. *Cyclamen hederifolium* (*C. hederifolium*) Ait. pripada familiji Primulaceae koja obuhvata 23 vrste ciklame, prirodno rasprostranjene u Centralnoj i Južnoj Evropi, Zapadnoj Aziji i nekim delovima Severne Afrike. Ta biljka se smatra otrovnom i nije pogodna za ljudsku upotrebu. Međutim, ekstrakti lukovica se koriste u tradicionalnoj medicini i homeopatiji. Cilj istraživanja je bio ispitivanje sadržaja metala, fenola, flavonoida i bioloških aktivnosti (antioksidativna, antibakterijska, antifungalna i citotoksična) *C. hederifolium* koji prirodno raste u Srbiji. **Metode.** Sadržaj metala određen je metodom atomske apsorpcione spektofotometrije. Za procenu antioksidativne aktivnosti i vodenog i etanolnog ekstrakta *C. hederifolium* korišćeno je više različitih testova: 2.2-*diphenyl-1-picrylhydrazyl* (DPPH) *radical scavenging assay*, 2,20-azino-bis (3-*ethylbenzothiazoline-6-sulfonic acid*) (ABTS) *radical scavenging assay*, *ferric-reducing antioxidant power* (FRAP) *assay*, *total reducing power* (TRP) *assay* i *cupric reducing antioxidant capacity* (CUPRAC) *assay*. Test disk difuzije je korišćen za ispitivanje osetljivosti laboratorijskih bakterijskih i gljivičnih sojeva na ispitivane ekstrakte. Vodeni i etanolni ekstrakti *C. hederifolium* ispitivani su na 4 različite tumorske ćelijske linije pomoću *in vitro* MTT testa. **Rezultati.** Prisustvo Mn, Ca,

Correspondence to: Ksenija Kojičić and Snežana Cupara, University of Kragujevac, Faculty of Medical Sciences, Department of Pharmacy, Svetozara Markovića 69, 34 000 Kragujevac, Serbia. E-mail: ksenija.kojicic@gmail.com, snezanacupara@yahoo.com

Mg, Fe, Zn, K, Cu je potvrđeno u vodenom i etanolnom ekstraktu, kao i u lukovicama i zemljištu, dok Cr, Ni, Pb i Cd nisu detektovani. I vodeni i etanolni ekstrakt lukovica *C. hederifolium* pokazali su antioksidativnu aktivnost, koja je bila u pozitivnoj korelaciji sa sadržajem fenola i flavonoida u njemu, pri čemu je vodeni ekstrakt bio superiorniji od etanolnog ekstrakta. Niti jedan od testiranih ekstrakata nije imao antimikrobnu aktivnost. Oba ekstrakta su ispoljila citotoksičnost protiv četiri tumorske ćelijske linije 4T1, HCT116, CT26, LLC1 u koncentracionom opsegu 15 625–2 000 μg/mL. Etanolni ekstrakt je pokazao snažniju citotoksičnost od vodenog ekstrakta. **Zaključak.** U zemlji, lukovicama i ekstraktima *C. Hederofolium* detektovano je sedam metala. Oba ekstrakta su pokazala antioksidativno i citotoksično dejstvo.

Ključne reči:
antioksidansi; ciklama; ekstrakti, biljni; metali; fitoterapija; spektrofotometrija, atomska apsorpciona.

Introduction

The number of newly diagnosed cancer cases as well as the percentage of deaths caused by cancer has been increasing in the world. As a result, many researchers focused attention on traditional herbal treatments, since it is an area from which a possible new antitumoral compound could arise. Investigators often search in local folk medicinal plant treatments of different regions, aiming to spot the possible anticancer activity of plants, since the current conventional (chemical) anticancer treatment has been accompanied by strong side effects [1, 2]. The studies on herbal material may reveal more than the desideratum. Various biological effects, such as antimicrobial, cytotoxic or antioxidant activity, have been often detected along the main investigation aim. This process that usually starts from *in vitro* or/and *in vivo* studies on herbal extracts, latter may or may not be rewarded by formulation of an efficient antitumor medicinal product. Nevertheless, it is a long, tedious, but often scientifically fruitful process. Therefore, we think that performing laboratory investigations as well as *in vitro* studies on plant material, especially on the species that are scarcely documented, is a first and prerequisite step on this path, regardless of current limitations in obtaining the final anticancer product [1, 2].

Cyclamen hederifolium (*C. hederifolium*) Ait. belongs to the family Primulaceae, which includes 23 species of cyclamen, naturally distributed in the Central and Southern Europe, Western Asia and some parts of North Africa. It is perennial, flowering plant, inhabiting partially shady woodlands and mountain meadows. There are several cultivated varieties and they differ in shape and color of flowers [3, 4]. Plant tubers of *C. hederifolium* are rich in toxic compounds which cause nausea, vomiting, diarrhea, abdominal pain, bloody stool, hemolysis, convulsions, skin irritation and blistering, and may even cause death due to the asphyxiation [3]. Though, the plant is considered highly poisonous and not suitable for human use, tubers have been used in homeopathy as well as in traditional medicine of different regions. *C. hederifolium* has been sporadically used in folk medicine in some parts of Serbia in the treatment of rheumatism, menstrual problems, migraine, and skin conditions, as a purgative and antitumor agent. Italian traditional medicine employs fresh tubers in the treatment of hemorrhoids and frostbites [3–7].

Several different biological activities have been noted for *Cyclamen* spp.*,* due to the presence of secondary metabolites produced by plant as key elements of its defense mechanism. There are also some reports on bioactivity of isolated compounds from the tubers of *Cyclamen* spp. [8, 9].

However, biological activity of *C. hederifolium* has not been abundantly documented. Among available data for *Cyclamen* spp. there are some results of *in vitro* studies on *C. hederifolium* that suggest cytotoxic, antioxidant, antiinflammatory and antimicrobial activity [6, 7, 10–14]. The molecular structure of compounds responsible for these effects have not been elucidated, but authors attributed some of biological activities of *C. hederifolium* to its content of phenols and triterpenic saponosides [8, 15, 16]. There are saponins in *C. hederifolium* that have been identified as cyclamin, deglucocyclamin, cyclaminorin, hederifoliosides A–E and ardisicrenoside D. After isolation they were investigated for inducing apoptosis, cytotoxic and hemolytic effects by the change of the cell membrane permeability and intracellular signaling pathways [8, 9, 14–16].

Therefore, the aim of this study was to complement available knowledge on *C. hederifolium,* by augmenting data on species from natural habitats in Serbia. The starting point was folk medicine of eastern parts of the country that utilizes this plant as an antitumor agent. We evaluated the metal content and biological activities that have not been previously reported for this species from natural habitats in Serbia – antioxidant, antimicrobial and cytotoxic activity of *C. hederifolium* tubers. Our intention was to clarify or justify the use of this plant by traditional medicine, which offers no documented data for antitumor use.

Methods

Plant material

Tubers of *C. hederifolium* were collected in September 2018 on the slopes of the mountain Suva Planina, belonging to the Eastern region of Serbia. The plant material was identified by standard botanical keys for plant determination (Flora of Republic of Serbia and European Flora) at Department of Biology at the Faculty of Science, University of Niš, Serbia [17, 18]. A voucher specimen no. 13556 was deposited in the "Herbarium Moesiacum Niš", University of Niš. Fresh *C. hederifolium* tubers were cleaned from the soil, separated from all other plant parts and dried in the shadow, at room temperature. Fresh tubers were used for extract preparation as well as for metal quantity analysis. Surrounding soil was sampled by collecting it from 10–50 cm area near tubers,

cleaned from stones and prepared for analysis in the shade at room temperature (20–25 ºC).

Extract preparation

The plant material was ground and mixed thoroughly with solvents. Two extracts were prepared by maceration of powdered plant material in two different solvents – distilled water and 70% v/v ethanol. Each extract was allowed to stand for five days at room temperature, in a closed glass container, protected from the light, and subject to frequent agitation. Excess solvent was evaporated at a rotary vacuum evaporator at 40 ºC. The dry obtained extracts were stored in a desiccator until the experiment.

Chemicals and instruments

2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS), iron (III) hloride hexahydrate, Folin–Ciocalteu reagent, gallic acid (3,4,5-trihydroxybenzoic acid), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), ascorbic acid, methanol, nitric acid, perchloric acid and all reagents used for cytotoxic assay were purchased from Sigma Chemicals Co. St. Louis, Missouri, USA. The chemical substances: neocuproine (2,9- dimethyl-1,10-phenanthroline), copper (II) chloride dihydrate, NaCO3, HCl, 2,4,2-tri(2-pyridyl)-s-triazine (TPTZ), K3[Fe(CN)6], phosphate buffer (NaH2PO4-Na2HPO4), ammonium acetate buffer, CCl3COOH, K2S2O8, FeSO4x7H2O, and DMSO (dimethyl sulphoxide) were purchased from Merck, Darmstadt, Germany. All chemicals were of analytical reagent grade. Absorbance measurement were performed on a double beam UV-Vis spectrophotometer Perkin Elmer lambda 15 (Massachusetts, USA) and microplate Zenyth 3100 Multimode detector (Anthos Labtec Instruments GmbH, Austria). Heavy metals were analysed by atomic absorption spectrophotometer (Perkin Elmer Company Model 3300/96 with MHS-10 hydride system).

Metal content determination

Comparative analysis of metal content was conducted in four different samples of *C. hederifolium tubers* – ethanol and aqueous extracts, fresh tubers and soil sample. Content of the following metals was determined by atomic absorption spectrophotometric method: Mn, Ca, Mg, Fe, Zn, K, Cu, Cr, Ni, Pb, Cd 19, 20. Determination of metal content in plant material was performed dissolving 1 g of sample by mixture of conc. HNO_3 and conc. H_2O_2. Mixture was heated. After cooling, 12 mL of distilled water was added and the mixture was filtrated. Determination of metal quantity in soil sample was performed after 1 g of sample was dissolved by 20 mL conc. HNO_3. The mixture was heated to 110ºC. After cooling, 2 mL conc. $HClO_4$ was added and the mixture was reheated to 130ºC. The heating was stopped when solution was evaporated to one third of the volume and clearing up of the solution was reached. After cooling, 12 mL of distilled water was added and the mixture was filtrated. Each measurement was performed in triplicate and results were expressed as mean (mg/kg) ± standard deviation.

Total phenolic and flavonoid content

Total phenolic content (TPC) was expressed as µg of gallic acid equivalents per mg of dry weight (µg GAE/mg dry extract). It was determined by Folin-Ciocalteu reagent, according to Singleton et al.[21]. Method is based on oxidation/reduction reaction. Briefly, 0.02 mL of the extract was mixed with 0.5 mL of Folin-Ciocalteu reagent, 5.03 mL diluted water and 2 mL sodium carbonate (20% Na_2CO_3). The mixture was allowed to stand in the dark for 30 min and absorbance was measured at 750 nm. Gallic acid (0.5 mg/mL) was used as standard. The experiments were carried as three independent measurements and data were expressed as mean ± standard deviation.

The total flavonoid content (TFC) was determined by the colorimetric method based on formation of complex between flavonoid and aluminium chloride, described by Baba and Malik[22]. Briefly, 0.05 mL of extract was mixed with 0.15 mL of 5% $NaNO_2$. After 5 min of incubation, 0.75 mL of 2% $AlCl_3$ was added, and the mixture was allowed to stand for 5 min. Then, 1 mL of NaOH and 2.05 mL of distilled water were added. The mixture was allowed to stand for 15 min. After expiration of incubation time, absorbance was measured at 520 nm. Rutin was used as standard. The total flavonoid content was calculated from a calibration curve, and the result was expressed as µg of rutin equivalents per mg dry weight (µg RE/mg dry extract). The experiments were carried as three independent measurements and data were expressed as mean ± standard deviation.

Antioxidant activity assays

We used several different assays for assessment of antioxidant activity of both aqueous and ethanol extracts of *C. hederifolium*: DPPH radical scavenging assay, ABTS radical scavenging assay, ferric-reducing antioxidant power (FRAP) assay, total reducing power assay (TRP) and cupric reducing antioxidant capacity (CUPRAC) assay.

DPPH radical scavenging capacity

The total antioxidant activity of the extracts was assessed by using 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay with slight modifications 23, 24. Method is based on the DPPH radical acceptation of H atom or electron from antioxidant molecules. DPPH radical changes the color from violet to yellow, when it is scavenged by reducing agents from extract and transformed to stable hydrazine. Absorbance was measured with spectrophotometer. Briefly, 1.5 mL of methanol solution of the DPPH radical (concentration 90 µmol/L), 0.002 mL of extract and 2.5 mL of methanol were placed in a test tube. Test tubes were allowed to stand at room temperature for 60 min in a dark place. The absorbance was measured at 515 nm against a

blank (methanol). All tests were carried out in triplicate and results were obtained from the calibration curve. Trolox in concentration 0.025 mg/mL was used as standard. Results are expressed as µg of Trolox equivalents per mg dry extract weight (µg TE/mg dry extract) and by EC-50 value which represents amount of extract necessary to scavenge the initial DPPH concentration by 50%.

ABTS radical scavenging activity

ABTS radical "scavenging" activity was performed followed the method of Dimitrijevic et al. [23], with some modifications. The working solution was prepared by mixing two stock solutions in equal quantities, 7 mM ABTS with 2.4 mM potassium persulfate. Blue/green ABTS radical was produced by the reaction of those solutions. Mixture was allowed to stand at room temperature in the dark for 12–16 h before use. The solution was then diluted by mixing 14.8 mL working solution with 240 mL of methanol. Absorbance was measured at 734 nm.

0.002 mL of plant extracts were mixed with 1.8 mL of diluted ABTS solution and then diluted with 2.198 mL of methanol. Mixture was allowed to stand for 6 min at room temperature. Antioxidants from extract cause discoloration of solution, proportional to their amount. The absorbance was measured at 734 nm. Trolox was used as standard and results are expressed as µg of TE per mg dry extract weight (µg TE/mg dry extract). The experiments were carried as three independent measurements and data were expressed as mean ± standard deviation.

Ferric-reducing antioxidant power (FRAP) assay

Antioxidant activity was also determined by the assay based on reduction of Fe^{3+}–TPTZ (tripyridyltriazine) complex to the intensive blue Fe^{2+}–TPTZ form at acidic pH 2.5. FRAP reagent was prepared by mixing 200 mL of $CH_3COONa \times 3H_2O$, 20 mL of TPTZ and 20 mL of $FeCl_3$ in ratio 10 : 1 : 1. After that, 0.01 mL of extract was mixed with 1 mL of prepared FRAP reagent and 2.99 mL of distilled water. Mixture was allowed to stand for 5 min at 37ºC. Absorbance was measured at 595 nm. The experiments were carried as three independent measurements and data were expressed as mean ± standard deviation. Results are expressed as µg Fe/mg dry extract.

Total reducing power (TRP) assay

The reducing power of water and ethanol *C. hederifolium* extracts was determined by the method of Oyaizu [26]. 0.01 mL of extract was mixed with 1 mL of 1% solution K3[Fe(CN)6], 1 mL phosphate buffer (pH 6.6) and 1.69 mL of distilled water. The mixture was allowed to stand at 50 ºC for 30 min. After expiration of the incubation time, 1 mL of 10% solution of CCl_3COOH and 0.6 mL of $FeCl_3$ were added. The absorbance was measured at 700 nm. Ascorbic acid was used as standard. The experiments were carried as three independent measurements and data were expressed as mean ± standard deviation. Results were expressed as µg ascorbic acid equivalents per mg of dry extract weight (µg AAE/mg dry extract). Higher absorbance indicates higher reducing power.

Cupric reducing antioxidant capacity (CUPRAC) assay

The CUPRAC assay was performed using the method based on electron-transfer mechanism described by Özyürek et al. [27], with some modifications. The method is based on the reduction of a cupric neocuproine complex (Cu(II)–Nc) by antioxidants to the cuprous form (Cu(I)–Nc). The CUPRAC method is applicable to a wide range of hydrophilic and lipophilic antioxidant molecules, such as polyphenol acids, flavonoids, carotenoids, anthocyanins, synthetic antioxidants and vitamin C and E 23, 28. Briefly, 0.01 mL of extract was mixed with 1 mL of neocuproine, 1 mL of phosphate buffer (pH 7.0) and 1 mL of copper (II) chloride. 1.09 mL of distilled water was added to the mixture. The mixture was allowed to stand for 30 min at room temperature. The absorbance was measured at 700 nm and Trolox (410 µg/mL) was used as standard. The experiments were carried as three independent measurements and data were expressed as mean ± standard deviation. Results are expressed as µg Trolox equivalents per mg of dry weight (µg TE/mg dry extract).

Antimicrobial activity

Disk diffusion assay

Both extracts of *C. hederifolium* (aqueous and ethanol) were examined for *in vitro* antimicrobial activity. The disk diffusion assay was used to investigate sensitivity of laboratory bacterial and fungal control strains (American Type Culture Collection – Maryland, USA) against investigated extracts [29, 30]. Initial concentration of extracts was 50 mg/mL. Bacterial strains were: Gram-positive bacteria *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538 and Gram-negative bacteria *Escherichia coli* ATCC 8739 and *Salmonella abony* NCTC 6017. *Candida albicans* ATCC 10231 was used as test strain for investigation of antifungal activity. Antibiotics streptomycin (10 µg/disk), chloramphenicol (30 µg/disk) and antimycotic nystatin (100 U/disk) were used as control substances. The experiments were carried as three independent measurements and data were expressed as mean ± standard deviation.

Evaluation of cytotoxicity

Cell culture

Both extracts (aqueous and ethanol) of *C. hederifolium* were examined on 4 different cell cultures (3 mouse and 1 human cancer cell lines) by *in vitro* 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) bioassay. Mouse lung cancer cells (LLC1), breast cancer cells (4T1), colon cancer cells (CT26) and human colon cancer cells (HCT116) were obtained from the American Type Culture Collection (ATCC). The cells were maintained in DMEM medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma, Germany), at 37 ºC in an atmosphere of 95% O2 and 5% CO2.

MTT bioassay

The investigated extracts of *C. hederifolium* were tested for *in vitro* cytotoxicity by standard colorimetric assay for measuring the cell viability (MTT test) [31]. Cancer cells were plated by adding 100 µL of media into each of the 96-well plates (Nunc A/S, Rockilde, Denmark). Plates were incubated at 37 ºC and 5% CO_2 overnight for adherence. After 24h, when the cells were attached to the surface of the plate, the medium was taken out and replaced with 100 µL of extracts, which had been serially diluted 2 fold in the medium to concentrations ranging 2,000 µg/mL, 1,000 µg/mL, 500 µg/mL, 250 µg/mL, 125 µg/mL, 62.5 µg/mL, 31.25 µg/mL and 15.625 µg/mL. Both extract was tested in triplicate, repeated in three independent series, and cisplatin was used as positive control. Plates were incubated at 37ºC in 5% CO_2 for 24 h, 48 h and 72 h. Cells were periodically viewed under fluorescence microscope Olympus BX51. After incubation periods the supernatant was removed, 100 µL MTT solution (5 mg/mL in PBS) in DMEM (Dulbecco's modified Eagle's medium) (10 µL MTT and 90 µL, per well) was added to each well. In this reaction, purple crystals of formazan were formed due to the reduction of yellow MTT by mitochondrial dehydrogenase of viable cells. After 4 h incubation under aforementioned conditions, the medium with MTT was removed. Then, DMSO (150 µL) with glycine buffer (20 µL) was added to each well in order to dissolve formazan crystals. The absorbance was measured at 595 nm using microplate Zenyth 3100 Multimode detector (Anthos Labtec Instruments GmbH, Austria). Results are presented as percentage of dead cells.

Data analysis

The experiments were carried as three independent measurements and data were expressed as mean ± standard deviation (SD). EC-50 values were calculated by using Microsoft Office Excel 2007.

Results

Metals found in all four investigated samples are presented in Table 1 (ethanol and aqueous *C. hederifolium* extracts, fresh plant tuber and soil). Cr, Ni, Pb and Cd were not detected in all samples, due to the concentrations that were below the detection limit.

Total phenolic content (TPC) and total flavonoid content (TFC)

Amount of phenols and flavonoids found in aqueous and ethanol extracts of *C. hederifolium* is presented in Table 2.

Antioxidant activity

The results of antioxidant activity of aqueous and ethanol *C. hederifolium* extracts, estimated by five different methods are shown in Table 3.

The comparison of the radical scavenging activity of investigated extracts measured by DPPH and ABTS assays expressed by EC-50 is presented in Figure 1.

Table 1

Metal content found in different *Cyclamen hederifolium* samples

Metal	Aqueous extract (mg/kg)	Ethanol extract (mg/kg)	Fresh tuber (mg/kg)	Soil (mg/kg)
Mn	157.32 ± 1.60	17.62 ± 0.41	47.42 ± 0.52	1141.56 ± 38.61
Ca	4018.8 ± 11.09	2263.86 ± 38.43	9053.4 ± 38.98	16748.98 ± 92.24
Mg	12052.36 ± 44.5	6770.78 ± 39.42	7256.06 ± 43.57	3162.62 ± 35.46
Fe	1250.2 ± 22.64	462.3 ± 11.85	375.06 ± 6.82	6448.66 ± 37.57
Zn	62.02 ± 1.07	23.96 ± 0.37	10.72 ± 0.40	37.44 ± 0.65
K	6826.04 ± 30.57	5224.8 ± 27.71	7070.12 ± 27.81	3265.34 ± 38.55
Cu	5.454 ± 0.06	5.542 ± 0.04	9.502 ± 0.05	9.904 ± 0.06

Table 2

Total content of phenols and flavonoids in *Cyclamen hederifolium* extracts

Extract	TPC (µg GAE/mg dw)	TFC (µg RE/ mg dw)
Aqueous	31.76 ± 2.92	28.17 ± 10.06
Ethanol	26.03 ± 1.32	23.2 ± 7.37

TPC – total phenolic content; TFC – total flavonoid content; GAE – gallic acid equivalents; RU – rutin equivalents.

Table 3

Antioxidant activity of *Cyclamen hederifolium* extracts (mean ± standard deviation)

Extract	DPPH µg TE/mg dw	FRAP µg Fe/ mg dw	ABTS µg TE/mg dw	TRP µg AAE/ mg dw	CUPRAC µg TE/ mg dw
Aqueous	8.153 ± 1.881	15.62 ± 0.576	6.21 ± 0.562	0.067 ± 0.008	5.893 ± 1.853
Ethanol	8.293 ± 1.167	12.57 ± 1.822	6.97 ± 0.534	0.05 ± 0.00	1.358 ± 1.160

DPPH – 2,2-diphenyl-1-picrylhydrazyl assay; FRAP – ferric-reducing antioxidant power assay; ABTS – 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay; TRP – total reducing power assay; CUPRAC – cupric reducing antioxidant capacity assay; TE – trolox equivalents; AAE – ascorbic acid equivalents.

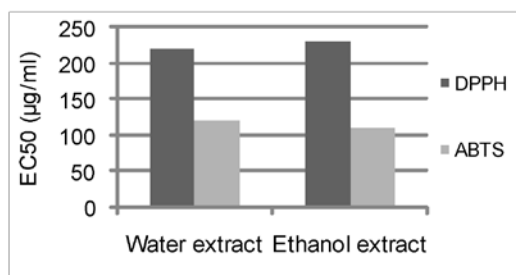


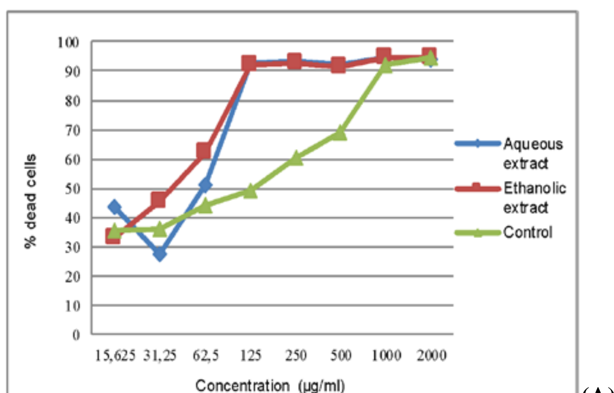
Fig. 1 – Free radical-scavenging of *Cyclamen hederifolium* extracts – DPPH vs. ABTS assay.
DPPH – 2,2-diphenyl-1-picrylhydrazyl assay; ABTS – 2,20-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) assay.

Antimicrobial activity

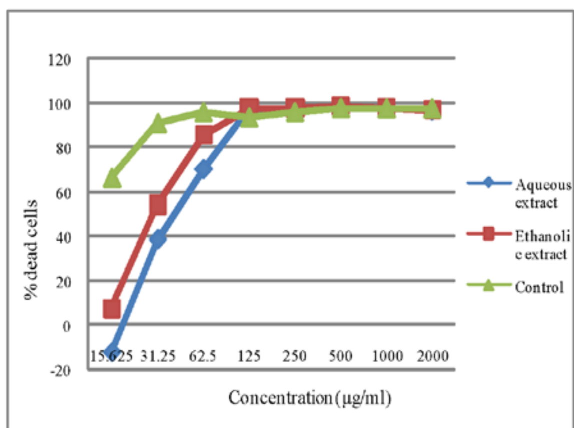
Antimicrobial activity of both investigated extracts of *C. hederifolium* against four bacterial and one fungal strain was negative.

Cytotoxic activity

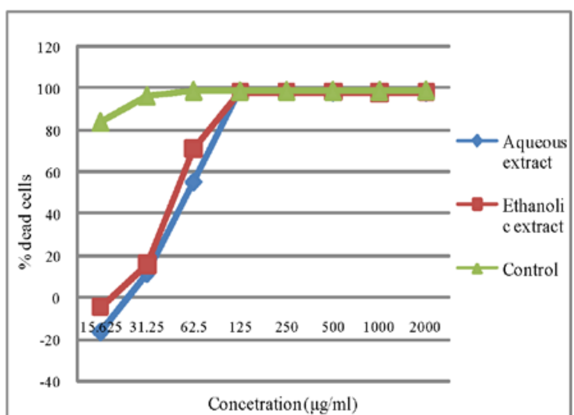
Results of the MTT assay show that both *C. hederifolium* extracts exhibit significant reduction of cell viability in all investigated carcinoma cells (Figures 2–5). Control was cisplatin.



(A)

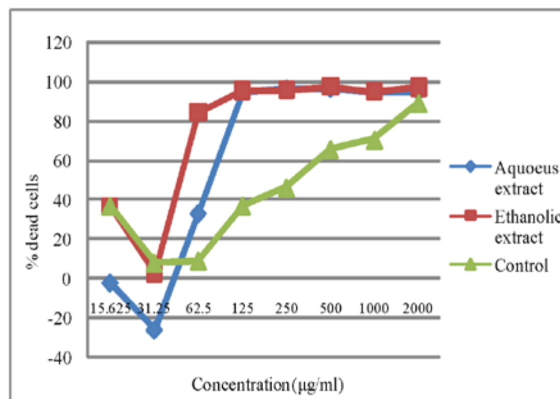


(B)

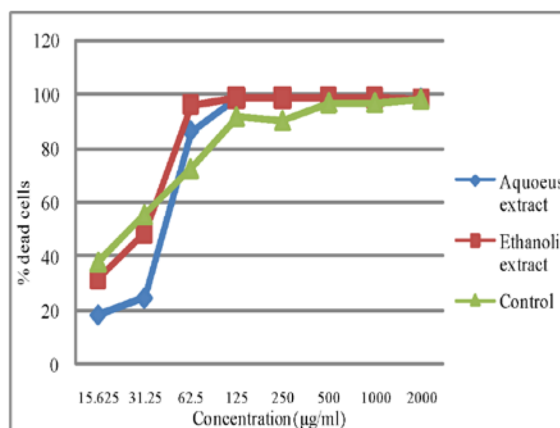


(C)

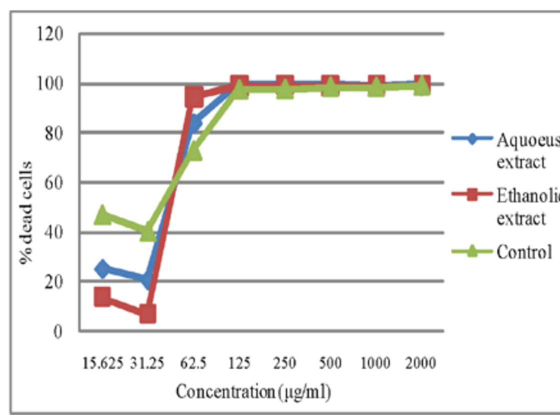
Fig. 2 – Mortality rate of 4T1 cell line exposed to aqueous and ethanolic extracts of *Cyclamen hederifolium* tuber during 24 h (A), 48 h (B) and 72 h (C).



(A)



(B)



(C)

Fig. 3 – Mortality rate of CT26 cell line exposed to aqueous and ethanolic extracts of *Cyclamen hederifolium* tuber during 24 h (A), 48 h (B) and 72 h (C).

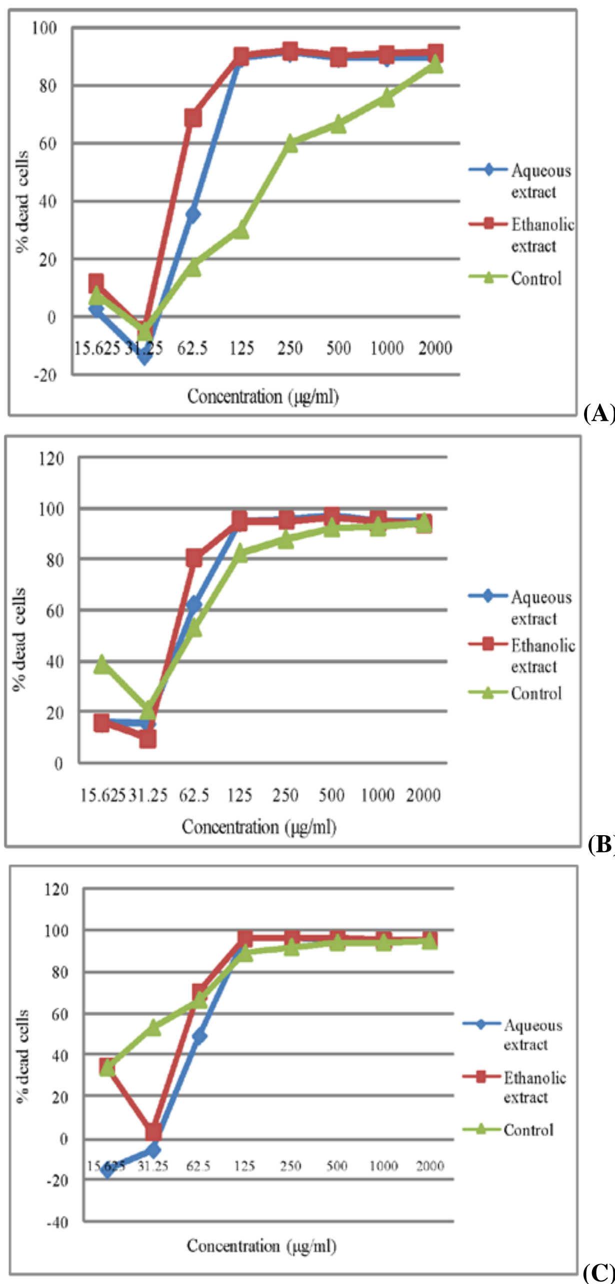


Fig. 4 – Mortality rate of HCT116 cell line exposed to aqueous and ethanolic extracts of *Cyclamen hederifolium* tuber during 24 h (A), 48 h (B) and 72 h (C).

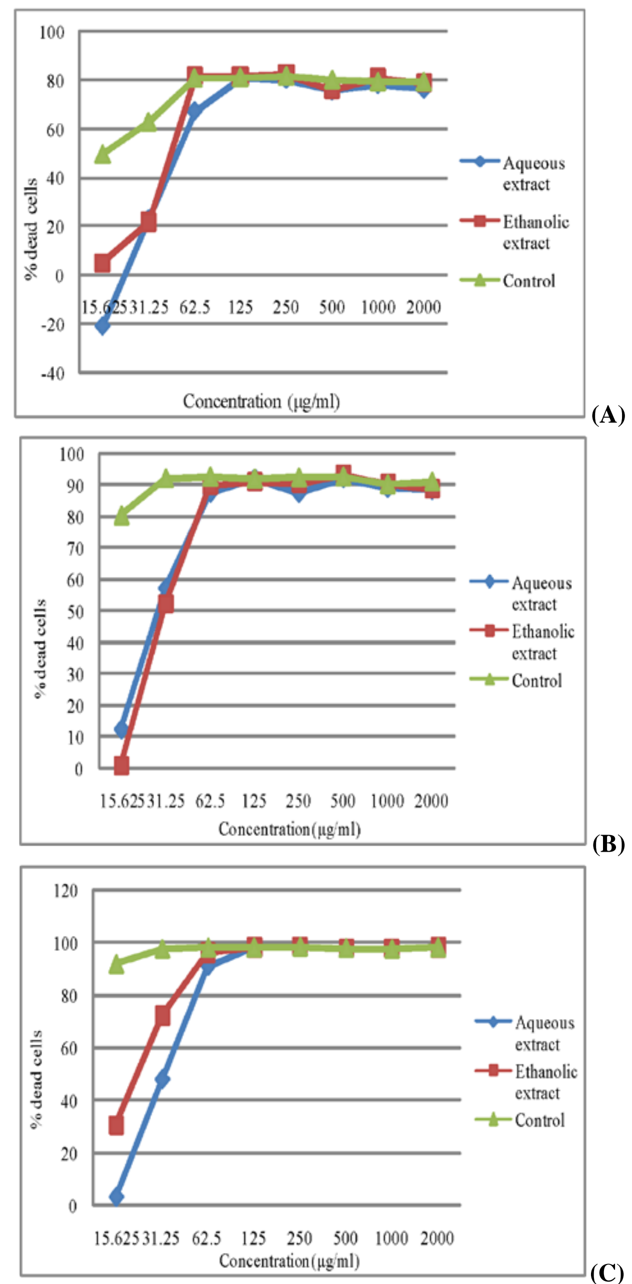


Fig. 5 – Mortality rate of LLC1 cell line exposed to aqueous and ethanolic extracts of *Cyclamen hederifolium* tuber during 24 h (A), 48 h (B) and 72 h (C).

Discussion

The tubers of *C. hederifolium* have been sporadically used in Serbian folk medicine in the treatment of different tumors. The absence of scientific evidence that supports use of *C. hederifolium* in this indication was a driving force to perform this study. Taking into account that most plants have been processed in folk medicine by water or alcohol (maceration, decoction, tinctures, etc.) and that have been no data regarding the bioactivity of *C. hederifolium* growing naturally in Eastern parts of Serbia, we investigated aqueous and 70% ethanol extracts for antioxidant, antibacterial, antifungal and cytotoxic properties, in order to evaluate the traditional application of this plant as anticancer agent.

Many plants have various uses due to their specific content of elements and secondary metabolites. Plants absorb water and minerals from soil where they are grown and store (accumulate) them in different parts. Many of micro and macro elements have an important role in human health, making monitoring of metal content plant material essential [32, 33]. The composition of minerals found in the plant material depends on species, parts of the plant, tendencies to take over and accumulate metals from soil, type of the growing environment (urban, industrial, rural, wilderness, etc.) and chemical composition of the soil [33, 34].

We followed metal content in four investigated samples in order to determine total quantity of metals present in *C.*

hederifolium fresh tubers and aqueous and ethanol extracts. Soil sample was monitored in order to determine if the plant is the accumulator of a specific mineral. The presence of Mn, Ca, Mg, Fe, Zn, K, Cu was confirmed in all four samples. Cr, Ni, Pb and Cd were not detected. Their below the limit concentrations are most likely related to unpolluted rural area where the tubers were collected from. Following metals were found in aqueous extract in the concentrations of the descending order: Mg > K > Ca > Fe > Mn > Zn > Cu, while ethanol extract contained: Mg > K > Ca > Fe > Zn > Mn > Cu. Aqueous extract had higher content of all observed metals than ethanol extract, except content of Cu which was similar in both extracts. Results of metal content analysis in relation to tuber, showed that aqueous extract had higher amount of Mn, Mg, Fe and Zn, while ethanol extract had more Fe and Zn. These findings indicate the difference in transfer of metals during extraction procedure. In this case, water has proven to be extraction solvent with higher potential for metal transfer than ethanol. Compared to soil, aqueous extract contained more Mg, Zn and K, while ethanol extract contained more Mg and K. The results demonstrated that the tuber samples contained higher quantity of Mg and K, in comparison to soil samples, which suggest potential phytoaccumulation of these metals [20].

The aqueous and ethanol extracts of *C. hederifolium* tubers were screened for composition of phenols and flavonoids, since these compounds have been mainly responsible for antioxidant activity of plants [23]. Aqueous extract contained slightly higher concentration of both phenols and flavonoids than ethanol extract. Several standard methods which were used to estimate antioxidant activity of investigated extracts (DPPH, FRAP, ABTS, CUPRAC and TRP assay) revealed that the aqueous extract of *C. hederifolium* exhibited stronger antioxidant activity than ethanol extract (FRAP and CUPRAC method). Therefore, we observe a positive correlation between TPC and TFC and antioxidant activity. Antioxidant activity of aqueous and ethanol extracts determined by DPPH, TRP and ABTS methods, however, did not confirm this difference. This absence of major differences between extracts in regards to the antioxidant activity as well as total phenol and flavonoid content could be attributed to predictable and small difference in polarity of solvents, which evidently exerted similar extraction potential.

There is generally a lack of the data in the literature on antioxidative activity of *C. hederifolium*. The only available information as regards antioxidative activity of *C. hederifolium* originated from Turkey – antioxidative activity of ethanol extracts of tubers was dose-dependent (by DPPH, ABTS and NO assays) [11]. We accentuate the results obtained in this study of *C. hederifolium* as the first information of phenol and flavonoid content and antioxidant activity of the plant material autochthon to this region.

Different *Cyclamen* species exhibited antimicrobial properties in varying degrees, such as *C. hederifolium* growing in Syria [31, 35, 36]. However, we may not compare our results to it, since extracts used in that research were made by different solvents that have no application in traditional medicine (methanol, petroleum ether, ethyl acetate and chloroform). The methanol extract showed better antimicrobial activities than the other extracts [6, 10]. This could be due to the differences in solvents polarity and their stronger ability to extract secondary metabolites responsible for antimicrobial activity. Aqueous and ethanol extracts of *C. hederifolium* tubers in our study showed absence of antimicrobial activity. We did not expected these findings, because some of secondary metabolites of *C. hederifolium* (e.g. saponins) in tubers may interfere with sterols in bacterial membrane and chaenge its integrity [9, 15, 16]. Since varying degrees of antimicrobial activity were detected for different types of *Cyclamen*, we consider that the limitation of this study was of number of strains that were tested as well as the selection of solvents used for extraction.

Some of previous studies documented that *Cyclamen* species may induce cell death in different tumor cell lines, although cytotoxic mechanisms have not been proposed [8, 13, 17]. Moderate cytotoxicity of *C. coum* was noted against cervical and lung cancer cells (HeLa and H1299, respectively) [35]. Moderate cytotoxic activity was reported for *C. trochopteranthum* against hepatocelular (HepG2) and colorectal adenocarcinoma (Caco-2) cell lines, while *C. pseudibericum* exhibited activity against nonsmall cell lung carcinoma cells (A549) [38, 39]. Although strong cytotoxic effect was observed in investigation of *C. libanoticum* and *C. persicum* against breast adenocarcinoma (SK-BR-3), colon adenocarcinoma (HT-29), hepatocelular (HepG2/3A), lung (NCI-H1299), pancreatic (BXPC-3), and prostate (22RV1) carcinoma cell lines, these effects could not be attributed to the extracts of these plants, since the experiments were performed with isolated and purified secondary metabolites (saponins) [8]. *C. hederifolum* was not tested on any of the cell lines we used in our investigation.

The effect of aqueous and ethanol extracts of *C. hederifolium* tubers on viability of several different cancer cell lines (4T1, HCT116, CT26, LLC1) were examined by MTT assay. Cell viability was tested after cell treatment with increasing concentrations of extracts, at different time of incubation (after 24 h, 48 h and 72 h). The results obtained in this study showed both aqueous and ethanol extracts of *C. hederifolium* exhibit cytotoxic activity in all investigated cell lines. Ethanol extract was superior in cytotoxic effect to aqueous extract, in all cell lines. Stronger cytotoxicity of both investigated extracts in comparison to control was observed in all carcinoma cell lines, in the first 24 h, with exception of LLC1 cell line. Concentrations lower than 125 μg/mL of both investigated extracts induced cell death up to 90%. However, higher concentrations of 125 μg/mL of both extracts showed an almost equal cytotoxic effect as control in all investigated cell lines, after 48 h and 72 h of incubation (90%–100%). The dose dependent manner of cytotoxcity was observed for different concentration in different cell line. Concentrations lower than 62.5 μg/mL for cell lines CT26 and LLC1 and concentrations lower than 125 μg/mL for 4T1 and HCT116, exerted dose dependent action. Higher concentration of extracts the for above mentioned cell lines reached a plateau in effectiveness (Figures 2–5). Unusual effect was observed for aqueous extract in concentrations up to 15.625 μg/mL, which produced no cytotoxic activity in some cell lines at different times of incubation – HCT116 and 4T1 cell line (24 h and 72 h of incubation) and CT26 (at 48 h and

72 h) and LLC1 (24 h). Based on these results, we might assume that ethanol as a solvent was more suitable for extraction of antitumor plant compounds than water.

To our knowledge, this is the first report for cytotoxic activity of endemic species *C. hederifolium* from natural habitats in Serbia. Although there were some previously reported data on cytotoxicity of *C. hederifolium*, there are major differences in methodology used in previous research and ours such as type of cytotoxic assay as well as the origin and preparation of the plant material (solvents used for the plant extraction and harvesting time of the plant material)[9, 12]. Both previous studies were done on plant material from Turkey. One study utilized plant material collected in autumn and evaluation of cytotoxicity by brine shrimp assay, while the other study investigated isolated compounds from *C. hederifolium* and reported no significant cytotoxicity. Therefore, we may not compare our results with aforementioned literature data which found low cytotoxic activity or absence of it in *C. hederifolium* from Turkey[9, 12]. Our results are however in accordance with other studies that point out the significant antitumor effects of *Cyclamen* spp. found on different cell lines[8, 34, 38, 39].

Since both investigated extracts showed cytotoxicity on four cancer cell lines (4T1, HCT116, CT26, LLC1) we consider this work as a starting point for further research, leading to elucidation of cell death mechanisms on proposed cell lines.

Limitations

The study was performed on 4 different tumor cell lines, but not on any control cell line. This is a potential limitation because it omitted the possibility of testing selectivity in cytotoxic activity of the examined extracts.

Conclusion

Seven metals have been identified in the *C. hederifolium* tubers, extracts and soil: Mn, Ca, Mg, Fe, Zn, K, Cu. Both aqueous and ethanol extract of *C. hederifolium* tubers exhibited antioxidant activity, which was positively correlated with content of phenols and flavonoids. Aqueous extract was slightly superior in these terms than ethanol extract. None of the tested extracts showed antimicrobial activity. Investigated extracts showed significant cytotoxicity in the concentration range 15.625–2,000 μg/mL. Ethanol extract showed stronger cytotoxic potential than aqueous extract. The reported data are the first data of this type for the species of *C. hederifolium*, with natural habitats in Serbia.

Acknowledgments

This work was supported by the projects No. OI 172051, No. OI 172047, and No. OI 175014 of the Ministry of Education, Science and Technological Development of the Republic of Serbia, as well as bilateral project with PR China (06/2018).

Conflict of interest

Authors declare that no conflict of interest exists.

REFERENCES

1. *De Vos P.* European material medica in historical texts: longevity of a tradition and implications for future use. J Ethnopharmacol 2010; 132(1): 28–47.
2. *Fürst R, Zündorf I.* Evidence-based phytotherapy in Europe: Where do we stand? Planta Med 2015; 81(12–13): 962–7.
3. *Grey-Wilson C.* Cyclamen: a guide for gardeners, horticulturalists and botanists. New edition. London: Batsford Ltd; 2003.
4. *Anderberg AA.* Phylogeny and subgeneric classification of Cyclamen L. (Primulaceae). Kew Bull 1994; 49(3): 455–67.
5. *Guarrera PM.* Traditional phytotherapy in Central Italy (Marche, Abruzzo, and Latium). Fitoterapia 2005; 76(1): 1–25.
6. *Mansour O, Rim S, Abbas L.* Screening of Antibacterial activity in vitro of Cyclamen hederifolium tubers extracts. Res J Pharm Tech 2016; 9(11): 1451–3.
7. *Mazouz W, Djeddi S.* A biological overview on the Genus Cyclamen. Eur J Sci Res 2013; 110(1): 7–22.
8. *El Hosry L, Di Giorgio C, Birer C, Habib J, Tueni M, Bun SS,* et al. In vitro cytotoxic and anticlastogenic activities of saxifragifolin B and cyclamin isolated from Cyclamen persicum and Cyclamen libanoticum. Pharm Biol 2014; 52(9): 1134–40.
9. *Altunkeyik H, Gulcemal D, Masullo M, Alankus-Caliskan O, Piacente S Karayildirim T.* Triterpene saponins from Cyclamen hederifolium. Phytochemistry 2012; 73(1): 127–33.
10. *Boissa NH, Yaziji M, Salame R.* Screening of antifungal activity in vitro of Cyclamen hederifolum tubers extracts. Res J Pharm Tech 2016; 9(10): 1677–80.
11. *Özay C, Ulukӧy G, Mammadov R, Sayin Z.* Radical scavenging activity and antibacterial effect of three Cyclamen L. tuber extracts on some fish pathogens. SDU J Nat App Sci 2018; 22(2): 562–8.
12. *Düşen S, Aydın C, Gul HY, Ozay C, Dusen O, Mammadov R.* In vitro cytotoxic activities of Cyclamen L. (Primulaceae) ethanol extracts from Turkey. Fresenius Envir Bull 2016; 25(12A): 6224–8.
13. *Yildiz M, Bozcu H, Tokgun O, Karagur ER, Akyurt O, Akca H.* Cyclamen exerts cytotoxicity in solid tumor cell lines: a step toward new anticancer agents? Asian Pac J Cancer Prev 2013; 14(10): 5911–3.
14. *Mohammed GJ, Hameed IH, Kamal SA.* Anti-inflammatory effects and other uses of Cyclamen species: A review. Ind J Pub Health Res Dev 2018; 9(3): 205–11.
15. *Koczurkiewicz P, Czyż J, Podolak I, Wójcik K, Galanty A, Janeczko Z,* et al. Multidirectional effects of triterpene saponins on cancer cells - mini-review of in vitro studies. Acta Biochim Pol 2015; 62(3): 383–93.
16. *Chudzik M, Korzonek-Szlacheta I, Król W.* Triterpenes as potentially cytotoxic compounds. Molecules 2015; 20(1): 1610–25.
17. *Nikolić V.* Genus Cyclamen L In: *Josifović M,* editor. Flora of SR Serbia III. Belgrade: Serbian Academy of Sciences and Arts; 1972. p. 500–2. (Serbian)
18. *Meikle DR, Sinnott HN.* Cyclamen L. In: *Tutin TG, Heywood WH, Burges NA, Moore DM, Valentine H, Walters SM,* et al, editors. Flora Europaea III. London, UK: Cambrige University Press; 1972. p. 25–6.
19. *Zlatić NM, Stanković MS, Simić ZS.* Secondary metabolites and metal content dynamics in Teucrium montanum L. and Teucrium chamaedrys L. from habitats with serpentine and calcareous substrate. Environ Monit Assess 2017; 189(3): 110.

20. *Brankovic S, Cupara S, Glisic R, Djelic G, Grbovic F, Kojicic K,* et al. Phytoaccumulation in plants of mountain GOC in Serbia. Studia Universitatis "Vasile Goldiş", Seria Ştiinţele Vieţii 2017; 27(3): 196–201
21. *Singleton VL, Orthofer R, Lamuela-Raventos RM.* Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Methods Enzymol 1999; 299(1): 152–78.
22. *Baba S, Malik S.* Determination of total phenolic and flavonoid content, antimicrobial and antioxidant activity of a root extract of Arisaema jacquemontii Blume. J Taibah Univ Sci 2015; 9(4): 449–54.
23. *Dimitrijevic M, Stankov Jovanovic V, Cvetkovic J, Mihajilov Krstev T, Stojanovic G, Mitic V.* Screening of antioxidant, antimicrobial and antiradical activities of twelve selected Serbian wild mushrooms. Anal Methods 2015; 7(10): 1–15.
24. *Tadić VM, Dobrić S, Marković GM, Dorđević SM, Arsić IA, Menković NR,* et al. Anti-inflammatory, gastroprotective, free-radical-scavenging, and antimicrobial activities of hawthorn berries ethanol extract. J Agric Food Chem 2008; 56(17): 7700–9.
25. *Benzie IF, Strain JJ.* The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power": the FRAP assay. Anal Biochem 1996; 239(1): 70–6.
26. *Oyaizu M.* Studies on Products of Browning Reactions: Antioxidative Activities of Product of Browning Reaction Prepared from Glucosamine. Jpn J Nutr 1986; 44(6): 307–15.
27. *Özyürek M, Güçlü K, Apak R.* The main and modified CUPRAC methods of antioxidant measurement. Trends Anal Chem 2011; 30(4): 652–64.
28. *Apak R, Güçlü K, Demirata B, Ozyürek M, Celik SE, Bektaşoğlu B,* et al. Comparative evaluation of various total antioxidant capacity assays applied to phenolic compounds with the CUPRAC assay. Molecules 2007; 12(7): 1496–547.
29. *Faleiro L, Miguel G, Gomes S, Costa L, Venâncio F, Teixeira A,* et al. Antibacterial and antioxidant activities of essential oils isolated from Thymbra capitata L. (Cav.) andOriganum vulgare L. J Agric Food Chem 2005; 53(21): 8162–8.
30. *Tomovic D, Bukonjic A, Jevtic V, Ratkovic ZR, Bogojeski JV, Dekovic A,* et al. DNA binding, antibacterial and antifungal activities of copper(II) complexes with some S-alkenyl derivatives of thiosalicylic acid. Transit Met Chem 2018; 43(2): 137–48.
31. *Tomovic DLJ, Bukonjic AM, Kocovic A, Nikolic MV, Mijajlovic MZ, Jevtic VV,* et al. Synthesis, characterization, and cytotoxicity of binuclear copper(II)-complexes with some S-alkenyl derivatives of thiosalicylic acid. Ser J Exp Clin Res 2017; 18(1): 13–8.
32. *Randjelovic S, Kostic D, Mitic S, Zarubica A, Mitic C, Velickovic J.* The correlation of metal content in medicinal plants and water their extract. Hem Ind 2013; 67(4): 585–91.
33. *Shah A, Niaz A, Ullah N, Rehman A, Akhlaq M, Zakir M,* et al. Comparative Study of Heavy Metals in Soil and Selected Medicinal Plants. J Chem 2013; ID 621265. doi: 10.1155/2013/621265.
34. *Grbovic F, Stankovic M, Vukajlovic F, Brankovic S, Simic Z, Topuzovic M.* Comparative study of the accumulation of metals in the plant Polygonum aviculare L. from different sites in the city of Kragujevac. Biologia Serbica 2016; 38(1): 3–11.
35. *Sajjadi ST, Saboora A, Mohammadi P.* Comparison of aglycon and glycosidic saponin extracts of Cyclamen coum tuber against Candida spp. Curr Med Mycol 2016; 2(2): 40–4.
36. *Turan M, Mammadov R.* Antioxidant, antimicrobial, cytotoxic, larvicidal and anthelmintic activities and phenolic contents of *Cyclamen alpinum.* J Pharm Pharmacol 2018; 9(4): 100–16.
37. *Alkowni R, Jodeh S, Hussein F, Jaradat N.* Phytochemical screening and antibacterial activity of *Cyclamen persicum* Mill tuber extracts. Pak J Pharm Sci 2018; 31(1): 187–92.
38. *Arslan S, Ozgun O.* Cyclamen Trochopteranthum: Cytotoxic activity and possible adverse interactions including drugs and carcinogens. Chin J Integr Med 2012; 1–8. Doi: 10.1007/s11655-012-1253-1.
39. *Karagur ER, Ozay C, Mammadov R, Akca H.* Antiinvasive effect of *Cyclamen pseudibericum* extract on A549 non-small cell lung carcinoma cells via inhibition of ZEB1 mediated by miR 200c. J Nat Med 2018; 72(3): 686–93.

Received on July 3, 2019.
Revised on September 5, 2019.
Accepted September 6, 2019.
Online First September, 2019.