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Cytotoxicity, antimicrobial and antioxidant activity of *Daucus carota* L., *Lycopersicon esculentum* Mill. and *Capsicum annuum* L.

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The objective of this study was to evaluate the cytotoxicity, antimicrobial and antioxidant activities of extracts of *Daucus carota L.*, *Lycopersicon esculentum* Mill. and *Capsicum annuum L.* grown in Central Serbia. The content of phenolic compounds in the extracts was determined using spectrophotometric and HPLC analysis. These plant extracts proved to be potent inhibitors of cell growth in the cell lines (FemX and LS 174). The IC50 values against FemX and LS174 cell lines were 33.52 ± 1.08 and 40.22 ± 0.03 μg/mL, respectively. The highest antioxidant capacity was found in *C. annuum L.* (98.01 ± 0.72 μg AA/g) and *D. carota L.* (48.51 ± 1.18 μg AA/g) extracts. The test extracts showed strong to moderate antimicrobial activity (19.53 - 312.50 μg/mL). *D. carota L., L. esculentum* Mill. and *C. annuum L.* extracts can serve as new dietary and food supplements.

Keywords: *Capsicum annuum L.; Daucus carota L.; Lycopersicon esculentum* Mill.; Cytotoxicity; Antimicrobial activity; Antioxidant activity.

INTRODUCTION

The medicinal properties of plants have been investigated in the light of recent scientific developments throughout the world, due to their potent pharmacological activities and low toxicity [1,2]. Antimicrobial activity of herbs has been known and described for several centuries [3]. Many naturally occurring compounds found in edible and medicinal plants, herbs, and spices have been shown to possess antimicrobial functions and could serve as a source of antimicrobial agents against bacteria and fungi [4,5,6].

Several studies have pointed out the possibility to use essential oils and/or their components in medical and plant pathology, as well as in food industry for the control of microorganisms pathogenic to consumers and/or responsible for food spoilage [7]. Everyday, our body is exposed to a large number of foreign chemicals [8]. Most of them are man-made and our inability to properly metabolize them negatively affects our health by generation of free radicals. Free radicals are also generated during normal metabolism of aerobic cells [9,10]. The oxygen consumption inherent to cell growth leads to the generation of a series of oxygen free radicals. Highly active free radicals and their uncontrolled production are responsible for numerous pathological processes such as cell tumor (prostate and colon cancers) and coronary heart diseases.

Antioxidants can significantly delay or prevent the oxidation of easily oxidizable substances [11]. According to their mechanism of action, natural antioxidants are classified as chain-breaking antioxidants which scavenge free radicals or inhibit the initiation step or interrupt the propagation step of lipid oxidation and as preventive antioxidants which slow the rate of oxidation by several actions but do not convert free radicals [12]. However, there have been concerns about synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) because of their possible activity as promoters of carcinogenesis [13]. Fruits and vegetables contain antioxidant compounds broadly called polyphenols that are known to reduce oxidative stress and prevent chronic diseases [14,15,16]. The antioxidant properties of these compounds are responsible for their anticancer, antiviral and antimicrobial properties. There is growing interest in natural antioxidants from plant sources [17].

The aim of this study was to evaluate the cytotoxicity, antimicrobial and antioxidant activities of extracts of *Daucus carota L., Lycopersicon esculentum Mill.* and *Capsicum annuum L.* grown in Central Serbia.

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MATERIALS AND METHODS

Chemicals

All standards for HPLC analysis were of analytical grade and were purchased from Sigma Chemical Co. (St Louis, MQ, USA) and Alfa Aesar (Karlsruhe, Germany). Acetonitrile and phosphoric acid were of HPLC grade (Tedia Company, USA). Ethanol was of analytical grade (Aldrich Chemical Co., Steinheim, Germany).

Plant Collection

The plants were cultivated under open field conditions during the autumn growing cycle in 2010 in an experimental field at Trbušani (Central Serbia).

Preparation of plant extracts

Plant samples (10.0 g) were extracted by 70% ethanol (100.0 ml) used as a solvent. The extraction process was carried out in an ultrasonic bath (Branson and Smith-Kline Company, B-220) at room temperature for 1 h. After filtration, 5 ml of the liquid extract was used for extraction yield determination. The solvent was removed by a rotary evaporator (Devarot, Elektromedicina, Ljubljana) under vacuum, and was dried to constant mass at 60°C. Dry extracts were stored in glass bottles at 4°C to prevent oxidative damage until analysis.

Determination of total phenolic content

Total phenols were estimated using the Folin-Ciocalteu method [18]. The extract was diluted to a concentration of 1 mg/ml, and aliquots of 0.5 ml were mixed with 2.5 ml of Folin-Ciocalteu reagent (previously diluted 10-fold with distilled water) and 2 ml of $NaHCO_3$ (7.5 %). The absorbance was measured at 765 nm using a UV/VIS spectrophotometer, after 15 min at 45˚C. Total phenols were determined as gallic acid equivalents (mg GAE/g extract), and the values were presented as means of triplicate analyses.

Determination of flavonoid content

Total flavonoids were determined according to [19]. A total of 0.5 ml of 2 % aluminium chloride ($AlCl_3$) in methanol was mixed with the same volume of the methanol solution of the plant extract. After storage for 1 h at room temperature, the absorbance was measured at 415 nm in a spectrophotometer against a blank sample. Total flavonoids were determined as rutin equivalents (mg RE/g dry extract), and the values are presented as means of triplicate analyses.

HPLC analysis

Quantification of individual phenolic compounds was performed by reversed phase HPLC analysis, using a modified method [20,21]. HPLC analysis was performed on an Agilent 1200 HPLC equipped with a diode array detector (DAD), Chemstation software (Agilent Technologies), a binary pump, an online vacuum degasser, an autosampler and a thermostated column compartment, on an Agilent, Zorbax Eclipse Plus-C18, 1.8 μm, 600 bar, 2.1×50 mm column, at a flow rate of 0.8 mL/min. Gradient elution was performed by varying the proportion of solvent A (methanol) to solvent B (1% formic acid in water (v/v)) as follows: initial 0-2 min, 100% B; 2-4 min, 100-98% B; 4-6 min, 98-95% B; 6-7 min, 95-73% B; 7-10 min, 75-48% B; 10-12 min 48% B; 12-20 min, 48-40% B. The total running time and post-running time were 21 and 5 min, respectively. The column temperature was 30ºC. The injection volume of samples and standards was 5 μL and injection was performed automatically using the autosampler. The spectra were acquired in the range 210–400 nm and chromatograms were plotted at 280, 330 and 350 nm with a bandwidth of 4 nm and a reference wavelength/bandwidth of 500/100 nm.

Cytotoxic activity

Cell lines

The human melanoma FemX and human colon carcinoma LS174 cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). Both cancer cell lines were maintained in the recommended RPMI-1640 medium supplemented with 10% heat-inactivated (56°C) fetal bovine serum, l-glutamine (3 mM), streptomycin (100 mg), penicillin (100 IU), and 25 mM HEPES and adjusted to pH 7.2 by bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Treatment of cell lines

Stock solutions (100 mg/mL) of extracts, made in dimethyl sulfoxide (DMSO), were dissolved in the corresponding medium to the required working concentrations. Neoplastic FemX cells (5000 cells per well) and neoplastic LS174 cells (7000 cells per well) were cultured in 96-well microtiter plates, and 24 h later, upon cell adherence, five different, double diluted concentrations of the test compounds were added to the wells. The final concentrations applied to the target cells were 200, 100, 50, 25 and 12.5 μg/mL, except to the control wells, where only nutrient medium was added to the cells. The nutrient medium was RPMI 1640 medium, supplemented with l-

glutamine (3 mM), streptomycin (100 lg/mL), and penicillin (100 IU/mL), 10% heat-inactivated (56°C) fetal bovine serum (FBS) and 25 mM Hepes, and was adjusted to pH 7.2 by bicarbonate solution. The cultures were incubated for 72 h.

Determination of cell survival (MTT test)

The effect of the extracts on cancer cell survival was determined by MTT test (microculture tetrazolium test) [22,23] 72 h upon addition of the compounds, as described earlier. Briefly, an aliquot of 20 µl of MTT solution (5 mg/mL PBS) was added to each well. The samples were incubated for further 4 h at 37ºC in 5% CO_2 and humidified air atmosphere. Then, 100 µL of 10% SDS were added to extract the insoluble product formazan resulting from the conversion of the MTT dye by viable cells. The number of viable cells in each well was proportional to the intensity of the absorbance of light, which was then read in an ELISA plate reader at 570 nm. Absorbance (A) at 570 nm was measured 24 h later. To obtain cell survival (%), A of a sample with cells grown in the presence of various concentrations of the test extracts was divided with the control optical density (A of control cells grown only in nutrient medium), and multiplied by 100. It was implied that A of the blank was always subtracted from A of the corresponding sample with target cells. IC50 concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. Cis-diamminedichloroplatinum (Cis-DDP) was used as a positive control. All experiments were done in triplicate.

Determination of antioxidant activity

The total antioxidant activity of the plant extracts was evaluated by the phosphomolybdenum method [23]. The assay is based on the reduction of Mo (VI) – Mo (V) by antioxidant compounds and subsequent formation of a green phosphate/Mo (V) complex at an acidic pH. A total of 0.3 ml of the sample extract was combined with 3 ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4mM ammonium molybdate). The tubes containing the reaction solution were incubated at 95°C for 90 min. Then, the absorbance of the solution was measured at 695 nm using a UV/VIS spectrophotometer (MA9523-SPEKOL 211, Iskra, Horjul, Slovenia) against a blank after cooling to room temperature. Methanol (0.3 mL) in the place of extract was used as the blank. Ascorbic acid (AA) was used as the standard and total antioxidant capacity was expressed as milligrams of ascorbic acid per gram of dry extract.

Determination of antimicrobial activity

Test microorganisms

The antimicrobial activity of the plant extract was tested *in vitro* against the following bacteria: *Staphylococcus aureus* ATCC 25923, *Klebsiella pneumoniae* ATCC 13883, *Escherichia coli* ATCC 25922, *Proteus vulgaris* ATCC 13315, *Proteus mirabilis* ATCC 14153, *Bacillus subtilis* ATCC 6633, and fungi: *Candida albicans* ATCC 10231 and *Aspergillus niger* ATCC 16404. The fungi were cultured on potato-glucose agar for 7 days at room temperature of 20 ºC under alternating light and dark conditions. They were recultured on a new potato-glucose substrate for another 7 days. The culturing procedure was performed four times until pure culture was obtained. The identification of the test microorganisms was confirmed by the Laboratory of Mycology, Department of Microbiology, Torlak Institute, Belgrade, Serbia.

Minimum inhibitory concentration (MIC)

Minimum inhibitory concentrations (MIC) of the extract and cirsimarin against the test bacteria were determined by the microdilution method in 96 multi-well microtiter plates [24]. All tests were performed in Muller–Hinton broth (MHB) with the exception of the yeast, in which case Sabouraud dextrose broth was used. A volume of 100 µL stock solutions of oil (in methanol, 200 µL/mL) and cirsimarin (in 10 % DMSO, 2 mg/mL) was pipetted into the first row of the plate. Fifty µL of Mueller Hinton or Sabouraud dextrose broth supplemented with Tween 80 at a final concentration of 0.5 % (v/v) for analysis of oil was added to the other wells. A volume of 50 µL from the first test wells was pipetted into the second well of each microtiter line, and then 50 µL of scalar dilution was transferred from the second to the twelfth well. Ten µl of resazurin indicator solution (prepared by dissolution of a 270-mg tablet in 40 mL of sterile distilled water) and 30 µL of nutrient broth were added to each well. Finally, 10 µL of bacterial suspension (106 CFU/mL) and yeast spore suspension (3×10^4 CFU/mL) was added to each well. For each strain, growth conditions and sterility of the medium were checked. The standard antibiotic amracin was used to control the sensitivity of the tested bacteria, whereas ketoconazole was used as control against the tested yeast. The plates were wrapped loosely with cling film to ensure that bacteria did not become dehydrated and were prepared in triplicate. Then, they were placed in an incubator at 37 °C for 24 h for the

bacteria and at 28 °C for 48 h for the yeast. Subsequently, color change was assessed visually. Any color change from purple to pink or colorless was recorded as positive. The lowest concentration at which color change occurred was taken as the MIC value. The average of 3 values was calculated, and the value obtained was taken as the MIC for the tested compounds and standard drug.

Statistical Analysis

The results are presented as mean ± standard deviations of three determinations. Statistical analyses were performed using Student´s t-test and one-way analysis of variance. Multiple comparisons of means were done by LSD (least significant difference) test. A probability value of 0.05 was considered significant. All computations were made by employing the statistical software (SPSS, version 11.0).

RESULTS AND DISCUSSION

Phenolic compounds of plant extracts

The total phenolic content and total flavonoids expressed as mg/g of dry extracts of *Daucus carota L.*, *Lycopersicon esculentum* Mill. and *Capsicum annuum L.* plants grown in an experimental field near Čačak (Central Serbia) are shown in Table 1.

The highest content of phenolic compounds was detected in the *D. carota* extract, 50.42 mg GAE/g. A similar content of total phenolic compounds was observed in the *L.esculentum* and *C. annuum* extract, about 30 mg GAE/g of dry extract. Flavonoids are phenolic compounds that have the highest antioxidant activity due to their chemical structure [25]. The lowest flavonoid content was found in the *L.esculentum* extract, 17.45 mg RE/g of dry extract. A higher and similar content of total flavonoid compounds was observed in *D. carota* and *C. annuum* extracts (around 23 mg RE/g) than in *L.esculentum* extracts.

In crude extracts, the following phenolic and hydroxycinnamic acids were identified and quantified using HPLC analysis: gallic acid, protocatechuic acid, caffeic acid, vanillic acid, chlorogenic acid, rosmarinic acid, ferulic acid, sinapic acid and syringic acid. Also, the flavonols: naringenin, myricetin, rutin and quercetin were identified (Figure 1. and Table 2.). The predominant components of *D. carota* extracts were chlorogenic acid and rosmarinic acid, while protocatehuic acid, sinapic acid and vanillic acid were found to be less dominant. The content of gallic acid, caffeic acid and ferulic acid was lower than 0.1 mg/g of dry extract. The predominant acids in *Lycopersicon esculentum* Mill. extract were gallic acid (0.37 mg/g) and caffeic acid (0.55 mg/g). The extract of *C. annuum* had the highest concentration of myricetin (3.32 mg/g).

Antioxidant capacity of plant extracts

Phenols and flavonoids play a dual role in reducing the rate of oxidation, as they participate in iron chelation and trapping radicals [26]. The antioxidant capacity of the ethanolic extracts of *Daucus carota L.*, *Lycopersicon esculentum* Mill. and *Capsicum annuum L.* is presented in Table 1. A strong correlation between free radical scavenging and the phenolic content has been reported for many plants. The highest antioxidant capacity was found in *C. annuum L.* (98.01 ± 0.72 μg AA/g) and *D. carota L.* (48.51 ± 1.18 μg AA/g) extracts. The ratio of total phenolic to total flavonoid content (TF/TP) in the *C. annum* extract was much higher than in the other two extracts, which is in agreement with literature data for these types of vegetables [27]. The effectiveness in reducing powers was in a descending order of *D. carota* > *L. esculentum* > *C.annum.*

D. carota extract shows the best antioxidant capacity and the highest content of phenolic compounds.

Table 1. Total phenols (TF), total flavonoids and their relationships (TF/TP), and antioxidant capacity of *Daucus carota* L., *Lycopersicon esculentum* Mill. and *Capsicum annum* L. ethanolic extracts*

Plant extracts	Total phenols (mg GAE/g d.e.)	Total flavonoids (mg RE/g of d.e.)	$\frac{TF}{TP}100$ [%]	Antioxidant capacity (μg AA/g d. e.)
D. carota L.	50.42 ± 0.02	23.19 ± 0.54	45.99	48.51 ± 1.18
L. esculentum Mill.	32.96 ± 0.65	17.44 ± 0.39	52.93	72.20 ± 0.72
C. annuum L.	33.34 ± 0.34	25.14 ± 0.32	75.41	98.01 ± 0.72

*Results are mean values ± SD from three experiments

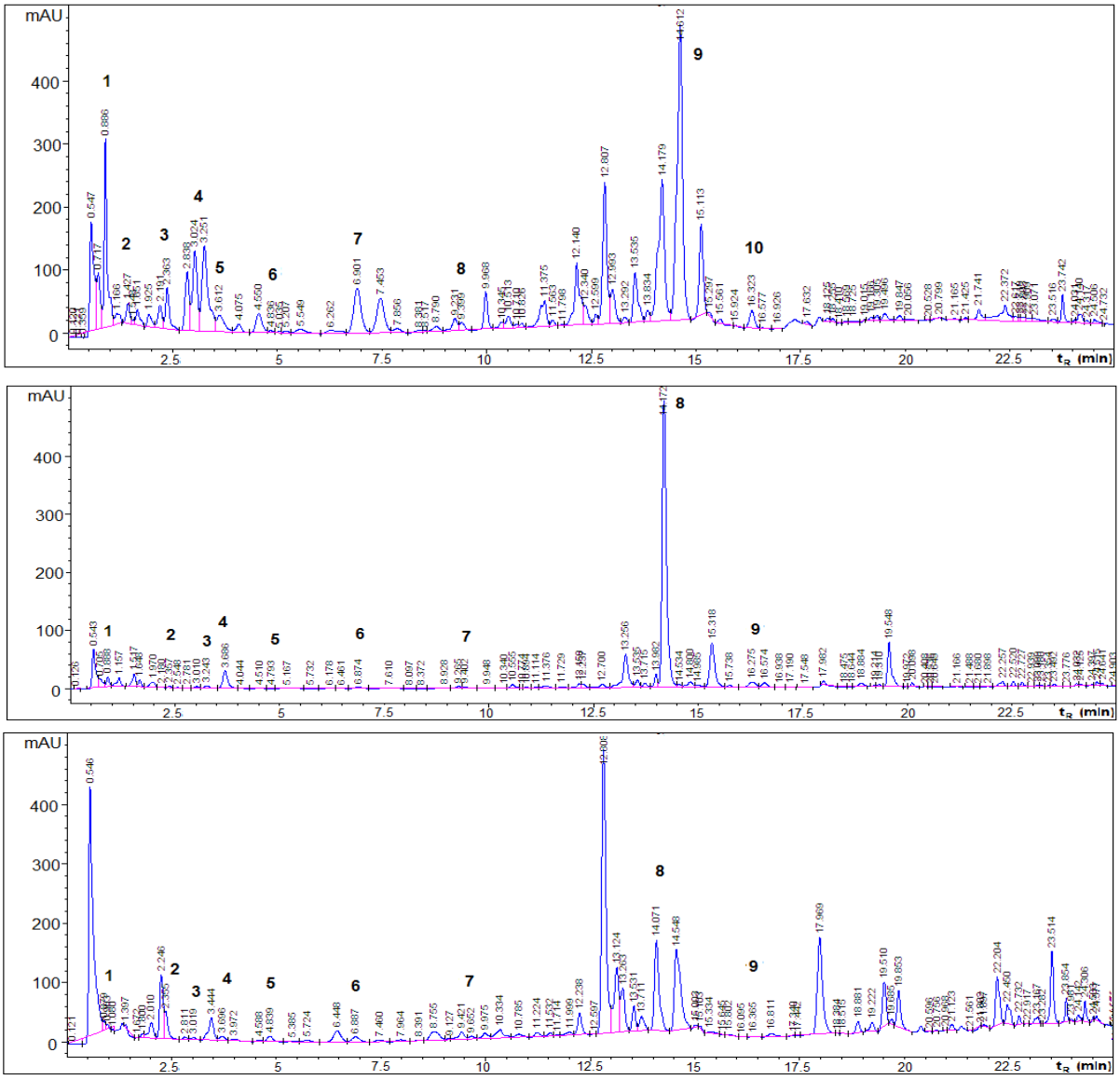

Fig. 1. HPLC chromatograms of the ethanolic extracts of *Daucus carota* L. (I), *Lycopersicon esculentum* Mill. (II) and *Capsicum annuum* L. (III)

Table 2. HPLC analysis of phenolic compounds of *Daucus carota* L. *Lycopersicon esculentum* Mill. and *Capsicum annuum* L. ethanolic extracts

	Phenolic compounds (mg/g of d. e.) of plant extracts		
	D. carota L.	*L.esculentum* Mill.	*C. annuum* L.
Gallic acid (1)*	0.09 ±0.01	0.37±0.02	0.17±0.01
Protocatechuic acid (2)	0.34±0.02	-	0.52±0.02
Caffeic acid (4)	0.06±0.02	0.55±0.02	-
Vanillic acid (5)	0.15±0.01	-	-
Chlorogenic acid (6)	0.80±0.02	-	-
Ferulic acid (9)	0.07±0.01	-	-
Sinapic acid (10)	0.25±0.01	-	-
Rosmarinic acid (13)	0.65±0.01	0.06±0.02	-
Syringic acid (8)	-	0.05±0.03	-
Cinnamic acid (14)	-	-	0.04±0.02
Myricetin (12)	-	-	3.32±0.01
Quercetin (15)	-	-	0.041±0.02
Naringenin (16)	-	-	0.02±0.01

*number corresponds to the peak

Cytotoxic activity of plant extracts

The tested extracts exhibited a strong cytotoxic activity against the target cells *in vitro* (Table 3.). The inhibitory concentration at 50% inhibition (IC50) was the parameter used to compare the cytotoxic activity. A lower IC50 meant better cytotoxic activity. *Daucus carota L*. showed the best cytotoxic activity. The IC50 values against FemX and LS174 cell lines were 33.52 ± 1.08 and 40.22 ± 0.03 μg/ml, respectively. The extract of *Lycopersicon esculentum* Mill. also showed good cytotoxic activity against both cell lines. The IC50 value was 43.51 ± 0.05 μg/mL against FemX cell and 30.29 ± 0.51 μg/ml against LS174 cell. *Capsicum annuum L*. exhibited slightly weaker cytotoxic activity. The IC50 value was 39.58 ± 0.38 μg/ml for FemX cell and 22.23 ± 0.95 μg/ml for LS174 cell.

Table 3. Growth inhibitory effects of the ethanolic extracts of *Daucus carota* L., *Lycopersicon esculentum* Mill. and *Capsicum annuum* L**.** on FemX and LS 174 cell lines

Plant extracts	IC_{50} (μg/mL)	
Daucus carota L.	33.52 ± 1.08	40.22 ± 0.03
L.esculentum Mill.	43.51 ± 0.05	30.29 ± 0.51
Capsicum annuum L	39.58 ± 0.38	22.23 ± 0.95
Cis-DDP	1.94 ± 0.55	7.41 ± 0.97

Antimicrobial activity of plant extracts

The results on minimum inhibitory concentrations (MIC) of the ethanolic extracts of *Daucus carota L., Lycopersicon esculentum* Mill. and *Capsicum annuum L.* for eight selected indicator strains are given in Table 4. The antimicrobial activity of the plants ranged from 19.53 μg/mL to 312.50 μg/mL. The extract of *D. carota* showed the highest antimicrobial susceptibility of the fungus *Aspergillus niger* (19.53 μg/mL). Intermediate antimicrobial susceptibility was exhibited by the yeast *Candida albicans* and the fungus *Aspergillus niger* to the *C. annuum* extract, by Bacillus subtilis to the *D. carota* extract and by *Bacillus subtilis* and the fungus *Aspergillus niger* to the *L.esculentum* Mill. extract (39.1 μg/mL). The lowest antimicrobial susceptibility was shown by *Staphylococcus aureus* to the *C. annuum* extract (312.5 μg/ml).

CONCLUSIONS

Intensive research on novel and natural antioxidant, antimicrobial and antitumor sources is highly important as it can lead to the creation of new pharmaceutical products. Finding natural sources of antioxidants can serve as an alternative to the use of toxic artificial antioxidants in the food industry. As shown by the results, the positive control (Cis-DDP) exhibited slightly better cytotoxic activity compared to the ethanolic extracts of *Daucus carota L., Lycopersicon esculentum* Mill. and *Capsicum annuum L.* This study suggests that all plant extracts show good antioxidant and antimicrobial properties and can serve as new dietary and food supplements.

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Table 4. Minimum inhibitory concentrations (MIC, μg/mL) of the ethanolic extracts of *Daucus carota* L., *Lycopersicon esculentum* Mill. and *Capsicum annuum* L.

Microbial strains	***C.annuum* L.**	***D. carota* L.**	*L.esculentum* Mill.	***Amracin***	***Ketoconazole***
	MIC (μg/ml)				
S. aureus ATCC 25923	312.50	156.25	156.25	0.97	/
K. pneumoniae ATCC 13883	156.25	78.13	78.13	0.49	/
E. coli ATCC 25922	78.13	78.13	78.13	0.97	/
P. vulgaris ATCC 13315	78.13	156.25	78.13	0.49	/
P. mirabilis ATCC 14153	156.25	78.13	156.25	0.49	/
B. subtilis ATCC 6633	78.125	39.10	39.10	0.24	/
C. albicans ATCC 10231	39.10	78.13	78.13	/	1.95
A. niger ATCC 16404	39.11	19.53	39.11	/	0.97

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ЦИТОТОКСИЧНОСТ, АНТИМИКРОБНО И АНТИОКСИДАНТНО ДЕЙСТВИЕ НА *DAUCUS CAROTA* L., *LYCOPERSICON ESCULENTUM* MILL. AND *CAPSICUM ANNUUM* L.

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(Резюме)

Целта на това проучване беше да се оцени цитотоксичността, антимикробната и антиоксидантната активност на екстракти от *Daucus Carota L.*, *Lycopersicon esculentum* Mill. и *Capsicum annuum L*. отгледани в централна Сърбия. Съдържанието на фенолни съединения в екстрактите се определяше чрез спектрофотометричен и HPLC анализ. Тези растителни екстракти се оказаха мощни инхибитори на клетъчния растеж в клетъчни линии (FemX и LS 174). Стойностите на IC50 срещу FemX и LS174 клетъчни линии бяха 33.52 ± 1.08 и 40.22 ± 0.03 мкг / мл, съответно. Най-високият антиоксидантен капацитет беше намерен в *C. Annuum L*. (98.01 ± 0.72 мкг АА / г) и *D. carota L.* (48.51 ± 1.18 мкг АА / г) екстракти. Тестваните екстракти показаха силна до умерена антимикробна активност (19.53 - 312.50 мкг / мл). Екстрактите от *D. carota L.*, *L. esculentum* Mill. и *C. annuum L*. могат да служат като нови диетични и хранителни добавки.