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Antioxidant and Biological Activities of Black Mulberry (*Morus nigra* L.) Fruit Depending on Altitude

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Abstract
This study evaluated the effect of altitude on the content of polyphenols (total phenolics, flavonoids, anthocyanins, condensed tannins and gallotannins), antioxidant activity (hydroxyl radical scavenging activity, inhibition of lipid peroxidation, and ABTS and DPPH assays), antimicrobial activity and cytotoxic activity in black mulberry (*Morus nigra* L.) fruit extracts. The research was conducted in western Serbia at three locations of different altitude: location 1—Katrga (187 m), location 2—Trbušani (271 m) and location 3—Miokovci (493 m). The results indicated that altitude had a significant impact on the accumulation and synthesis of the tested parameters. At higher altitudes, the accumulation of total phenolics, flavonoids and anthocyanins increased. The highest values were determined at the location situated at an altitude of 493 m. By contrast, the accumulation of condensed tannins and gallotannins was most intensive at the lowest-altitude location (187 m). Microbial properties of extracts were examined using eight selected indicator strains. Minimum inhibitory concentrations varied from 19.53 to 156.25 µg/mL, depending on altitude. The fruit of black mulberries grown at 187 m had the highest antioxidant and cytotoxic activity compared to altitudes of 271 m and 493 m altitudes. The concentrations of extracts which inhibited 50% of cell growth for Hep2c, RD and L2OB at the lowest altitude were 18.55, 15.32, and 37.17 µg/mL, respectively. The present results showed that altitude had a significant effect on the synthesis and accumulation of polyphenols, and a strong effect on the antioxidant, antimicrobial and cytotoxic activities of black mulberry fruit.

Keywords Black mulberry · Altitude · Biochemical composition · Antimicrobial · Cytotoxic activity

Introduction

In Serbia, black mulberry (*Morus nigra* L.) fruits are used minimally in human diet, due to scarce data in the literature on their phytochemical composition and medicinal properties. However, in recent years, black mulberries have been the subject of numerous studies on detailed chemical characterization of berries and their use in the nutritional and pharmaceutical industries as a source of new biological ingredients. Black mulberry fruits are a rich source of ascorbic acid, phenolics and flavonoids, including anthocyanins, which belong to naturally occurring phenolic compounds responsible for the colour of mulberries. High levels of phenolic compounds and anthocyanins in berry extracts demonstrate high antioxidant, antimicrobial and anti-inflammatory activities (Kim and Park 2006; Isabelle et al. 2008). Kostić et al. (2013) reported that black mulberry fruits from Serbia have a high content of natural phenolic compounds as well as anthocyanins, and exhibit high antioxidant activities. The polyphenolic composition of black mulberries is significantly influenced by genotype, climatic conditions (precipitation, temperature, altitude), soil, length of the growing season, and light intensity (Uleberg et al. 2012; Guerrero-Chavez et al. 2015; Gündüz and Özbay 2018). Altitude has rarely been assessed in relation to its impact on polyphenolic composition and fruit quality. Changes in altitude are always associated with changes in environmental conditions, such as temperature, precipitation, light intensity etc., which directly influence the metabolism of plants and the concentration of various compounds in berries. As emphasized by Dong et al. (2011) and Zoratti et al. (2015), along with temperature, light intensity and moisture, altitude is an important factor influencing the accumulation and synthe-

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sis of secondary metabolites and the resulting quality and commercial value of fruits.

Therefore, the objective of this study was to determine the effects of different altitudes on the accumulation and synthesis of bioactive polyphenols, as well as on the antioxidant, antimicrobial and cytotoxic activities of fruit extracts of black mulberries.

Materials and Methods

Plant Material

The research was conducted in western Serbia, during 2017–2019, at three locations of different altitude: location 1—Katrga (43°49′ N latitude, 20°33′ E longitude, 187 m altitude), location 2—Trbušani (43°55′ N latitude, 20°19′ E longitude, 271 m altitude) and location 3—Miokovci (43°58′ N latitude, 20°14′ E longitude, 493 m altitude). Growing conditions for black mulberries across locations were uniform. Fruits were sampled at full ripeness in June from similar portions of the trees.

Sample Preparation

Fruit samples (10.0 g) were extracted with 96% ethanol (100.0 mL) as a solvent. They were extracted with an ethanol:water:acetic acid (70:29.5:0.5, v/v/v) mixture, at a temperature of 30 °C, at the same liquid–solid ratio. The extraction process was carried out using an ultrasonic bath (model B-220, Branson Instruments, Smith-Kline Co., Danbury, CT, USA) at room temperature for 1 h. After filtration, 5 mL of the liquid extract was used to determine the extraction yield. The solvent was removed using a rotary vacuum evaporator (Devarot, Elektromedicina, Ljubljana, Slovenia) and dried at 30 °C until constant mass was achieved. The dried extracts were stored in glass bottles at 4 °C to prevent oxidative damage until analysis.

Determination of Polyphenol Composition

1. Total phenolics were determined spectrophotometrically via the Folin–Ciocalteu method (Singleton et al. 1999). The reaction mixture was prepared by mixing 0.5 mL of the methanolic solution of the extract, 2.5 mL of 10% Folin–Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% $NaHCO_3$, and allowed to react at room temperature over 1 h. A blank was concomitantly prepared and contained 0.5 mL methanol, 2.5 mL of 10% Folin–Ciocalteu's reagent dissolved in water and 2.5 mL of 7.5% NaHCO3. The absorbance was measured at 765 nm. Gallic acid was used as the standard reagent. Results are expressed as milligrams per gram of dry extract (mg GA g^{-1} DW).
2. The total flavonoid content of the extract was estimated using a colorimetric assay based on the procedure described by Markham (Markham 1989). The extract solution (10 mg/mL, 1 mL) was mixed with a 5% sodium nitrite solution (0.3 mL). After 5 min, aluminium chloride (10%, 0.3 mL) was added and allowed to stand for a further 6 min. Then, sodium hydroxide (1 mol/L, 1 mL) was added to the mixture. Immediately thereafter, distilled water was added to the final volume of 10.00 mL. A blank was prepared using water instead of samples. The absorbance was read at 415 nm and rutin was used as the reference standard. Results are expressed as milligrams per gram of dry extract (mg GA g^{-1} DW).
3. The method for the determination of condensed tannins relies on the precipitation of proanthocyanidins with formaldehyde (Verrmeris and Nicholson 2006). The concentration of condensed tannins was calculated as the residue of the total phenolic and unprecipitated phenol concentrations, and expressed as gallic acid equivalents. Results are expressed as milligrams per gram of dry extract (mg GA g^{-1} DW).
4. Gallotannins were determined using the potassium iodate assay (Verrmeris and Nicholson 2006), which is based on the reaction of potassium iodate with galloyl esters. The reaction was performed by adding 1.5 mL of a saturated potassium iodate solution to 3.5 mL of extract at a temperature over 40 °C until maximum absorbance was obtained. The concentration of the produced red intermediate was measured spectrophotometrically at 550 nm and gallotannin content was determined using gallic acid equivalents. Results are expressed as milligrams per gram of dry extract (mg GA g^{-1} DW).
5. Total anthocyanins were quantified by the single pH and pH differential method. An aliquot of 0.25 mL of the extracted sample was dissolved in 0.25 mol L^{-1} of KCl buffer at pH 1.0 and 0.4 mol L^{-1} of CH_3COONa buffer at pH 4.5. After 15 min, the absorbance was measured at 515 nm and 700 nm. Results are expressed as milligrams of cyanidin-3-glucoside equivalents per gram of fresh weight (mg C3G g^{-1} FW).

Determination of Antioxidant Activity

The antioxidant activity of fruit extracts was assessed using four different assays:

1. The capacity to scavenge the "stable" free radical DPPH was monitored according to the method of Takao et al. (1994) adopted with suitable modifications from Kumarasamy et al. (2007). Various concentrations of the extract (2 mL) were mixed with DPPH (80 µg/mL, 2 mL).

The mixture was incubated at room temperature for 30 min and the absorbance was measured at 517 nm. Methanol was used to set zero of absorbance. Ascorbic acid, gallic acid and butylated hydroxytoluene were used as reference standards.

2. The ability to inhibit non-site-specific hydroxyl radical-mediated peroxidation was carried out according to Hinneburg et al. (2006). Extract was dissolved in water to a final concentration of 1 mg/mL. Sample solution was further mixed with 500 µL of 5.6 mmol/L 2-deoxy-D-ribose in sodium phosphate buffer (50 mmol/L, pH = 7.4), 200 µL of premixed 100 µM of iron (III) chloride and 104 mmol/L EDTA (1:1 v/v) solution, 100 µL of 1.0 m MH_2O_2, and 100 µL of 1.0 mM aqueous ascorbic acid. Reaction mixture was incubated at 50 °C for 30 min. Thereafter, 1 mL of 2.8% trichloroacetic acid and 1 mL of 1.0% thiobarbituric acid were added to each tube. The samples were vortexed and heated in a water bath at 50 °C for 30 min. The extent of oxidation of 2-deoxyribose was estimated from the absorbance of the solution at 532 nm. The percentage inhibition values were calculated from the absorbance of the control (*A*c) and of the sample (*A*s), where the controls contained all the reaction reagents except the extract or positive control substance.
3. Lipid peroxidation inhibitory activity was determined using the thiocyanate method (Hsu et al. 2008). Extract was dissolved in water to a final concentration of 1 mg/mL. This solution was further used for the preparation of serial dilutions, and 0.5 mL of each solution was added to linoleic acid emulsion (2.5 mL, 40 mM, pH = 7.0). The linoleic acid emulsion was prepared by mixing 0.2804 g of linoleic acid and 0.2804 g of Tween-20 in 50 mL of 40 mM phosphate buffer. The mixture was incubated at 37 °C for 72 h. Thereafter, 0.1 mL of the reaction solution was mixed with 4.7 mL of ethanol (75%), 0.1 mL of iron (II) chloride (20 mmol/L), and 0.1 mL of ammonium thiocyanate (30%). The absorbance of this mixture was measured at 500 nm, and the mixture was stirred for 3 min. α-Tocopherol, gallic acid, ascorbic acid and butylated hydroxytoluene were used as reference compounds.
4. The antioxidant capacity was estimated in terms of the $ABTS^{\bullet+}$ (2, 2'-azino-bis (3 ethylbenzothiazoline-6-sulphonic acid)) scavenging activity following the procedure described by Delgado-Andrade et al. (2005). Briefly, $ABTS^{\bullet+}$ was obtained by reacting 7 mM ABTS stock solution with 2.45 mM potassium persulfate and the mixture was allowed to stand in the dark at room temperature for 12–16 h before use. The $ABTS^{\bullet+}$ solution was diluted with 5 mM phosphate-buffered saline (pH = 7.4) to an absorbance of 0.70 ± 0.02 at 730 nm. After the addition of 10 µL of sample to 4 mL of diluted $ABTS^{\bullet+}$ solution, the absorbance was read after 30 min. BHT, GA and AA were used as reference antioxidants. All tests were performed in triplicate and the results are expressed as IC50 values.

Determination of Antimicrobial Activity

Antimicrobial activity was tested in vitro against the following bacteria: *Staphylococcus aureus, Klebsiella pneumoniae, Escherichia coli, Proteus vulgaris, Proteus mirabilis, Bacillus subtilis*; and the fungi *Candida albicans* and *Aspergillus niger*. Minimum inhibitory concentrations of the extracts and cirsimarin against the test bacteria were determined by microdilution method in 96-multi-well microtiter plates (Satyajit et al. 2007). All tests were performed in Mueller–Hinton broth except for 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) were pipetted into the first row of the plate. Fifty microliters of Mueller–Hinton or Sabouraud dextrose broth (supplemented with Tween 80 to a final concentration of 0.5% (v/v)) 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 12th well. Then, 10 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 (10^6 CFU/mL) and yeast spore suspension (3×10^4 CFU/mL) were added to each well. For each strain, the growth conditions and the sterility of the medium were checked. Standard antibiotic amracin was used to control the sensitivity of the tested bacteria, whereas nystatin was used as a control against the tested yeast. Plates were wrapped loosely with cling film to prevent dehydration, and were prepared in triplicate. The plates 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, colour change was assessed visually. Any colour change from purple to pink or colourless was recorded as positive. The lowest concentration at which colour change occurred was taken as the MIC value.

Determination of Cytotoxic Activity

The influence on growth of malignantly transformed cell lines was evaluated by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) assay. The following cell lines were used: RD (cell line derived from human rhabdomyosarcoma), Hep2c (cell line derived from human cervix carcinoma—HeLa derivative) and L2OB (cell line derived from murine fibroblast). Using 96-well cell culture plates, the cells were seeded in nutrient medium (minimum essential medium—MEM) and grown at 37 °C in a humidified atmosphere for 24 h. The corresponding extracts

and control (absolute ethanol) were diluted with nutrient medium to desired concentrations. Diluted samples and control were added to the cells (100 lL per well). The cells were incubated at 37 °C in a humidified atmosphere for 48 h. After incubation, supernatants were discarded, MTT (stock solution: 5 mg/mL in PBS) dissolved in D-MEM medium to a final concentration of 500 µg/mL was added to each well (100 µL/well), and plates were incubated at 37 °C in humidified atmosphere for 4 h. Reactions were stopped by the addition of 10% SDS/10 mM HCl (100 µL/well). After overnight incubation at 37 °C, absorbances were measured at 580 nm using a spectrophotometer (Ascent 6–384 [Suomi], MTX Lab Systems Inc., Vienna, VA, USA). The number of viable cells per well (NVC) was calculated from a standard curve plotted as cell numbers against A580. Corresponding cells (grown in flasks), after cell count with a haemocytometer, were used as standards. Standard suspensions were plated in serial dilution, centrifuged at 800 rpm for 10 min and then treated with MTT/D-MEM and 10% SDS/10 mM HCl solutions in the same way as the experimental wells (ut supra). The number of viable cells in each well was proportional to the intensity of the absorbed light, which was then read in an ELISA plate reader. Absorbance at 580 nm was measured 24 h later. Cell survival (%) was calculated by dividing the absorbance of a sample with cells grown in the presence of various concentrations of the investigated extracts with control optical density (the A of control cells grown only in nutrient medium), and multiplying by 100. The IC50 concentration was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. The results of the measurements are expressed as the percentage of positive control growth taking the *cis*-diamminedichloroplatinum (*cis*-DDP) determined in positive control wells as 100% growth (Dighe et al. 2011; Baviskar et al. 2012).

Statistical Analysis

The experimental data were subjected to statistical analysis using Fisher's two-factor analysis of variance (ANOVA). Significant differences between the mean values of the tested factors and the interaction means were determined by LSD test at $p \leq 0.05$ significance levels.

Results and Discussion

Various factors, such as genotype, location and climatic conditions, contribute to phenolic profiles of plants (Mikulic-Petkovsek et al. 2012). One of these factors is altitude, which has not been sufficiently investigated to date. Only a few studies deal with altitude and its impact on secondary metabolites in fruits, especially with regard to the content of phenolic compounds. The results on the effect of altitude on polyphenols in black mulberry fruit extracts are presented in Table 1. In the present study, the content of total phenolics in fruit extracts ranged from 13.01 to 13.99 mg GAE g^{-1}, and flavonoids from 3.12 to 3.87 mg GAE g^{-1}. Detected amounts of anthocyanins varied from 2.35 to 2.74 mg C3G g^{-1}, while the measured concentration of condensed tannins ranged from 4.41 to 4.57 mg GA g^{-1} and gallotannins from 2.19 to 2.38 mg GA g^{-1}.

Table 1 Contents of polyphenols in black mulberry fruit

	Location 1 (187 m a.s.l.)	Location 2 (271 m a.s.l.)	Location 3 (493 m a.s.l.)
Total phenolics (mg GA g^{-1})	13.01 ± 1.43 c	13.71 ± 1.90 b	13.99 ± 1.28 a
Flavonoids (mg RU g^{-1})	3.12 ± 0.96 c	3.54 ± 0.31 b	3.87 ± 0.82 a
Anthocyanins (mg GA g^{-1})	2.35 ± 0.69 c	2.56 ± 0.36 b	2.74 ± 0.71 a
Condensed tannins (mg GA g^{-1})	4.57 ± 0.90 a	4.41 ± 0.87 b	4.47 ± 0.86 b
Gallotannins (mg GA g^{-1})	2.38 ± 0.43 a	2.26 ± 0.36 b	2.19 ± 0.38 b

Means followed by different letters within rows are significantly different at $p \leq 0.05$ according to LSD test and ANOVA (*F* test) results

Compared to the present study, Ercisli and Orhan (2007) and Okatan (2018) reported a higher content of total phenolic and flavonoid, whereas Radojković et al. (2012) reported similar values for total phenolic, flavonoid and anthocyanins. The present results showed that the synthesis of polyphenols in black mulberry fruit extracts was affected by altitude. The accumulation of total phenolics, flavonoids and anthocyanins increased with increasing altitude. The highest values of the tested parameters were determined at the location situated at 493 m. By contrast, the accumulation of condensed tannins and gallotannins was higher at the lowest-altitude location (187 m). It can be assumed that differences in the studied parameters were due to a combination of climatic factors (temperature, humidity, precipitation etc.), which affected the synthesis and accumulation of polyphenols at different altitudes. These results are supported by the findings of Berli et al. (2008), who observed that grapes grown at 1500 m contained a higher amount of total polyphenols and anthocyanins compared to berries grown at 1000 m and 500 m. As reported by Zoratti et al. (2015), the accumulation of anthocyanins in wild bilberries and highbush blueberries was higher with increasing altitude, and seasonal differences, especially temperature, had a major influence on the accumulation of anthocyanins. The content of anthocyanins, according to Uleberg et al. (2012), is greatly affected by environmental factors (light intensity, light quality and temperature), which was taken into con-

sideration when explaining the accumulation of metabolites in bilberry fruits. Also, Martz et al. (2010) indicated that plants growing at higher altitudes accumulate more than twofold levels of total phenolics compared with plants growing at lower altitudes. Namely, Spinardi et al. (2009) reported that higher altitudes increased total anthocyanins by 50% and total phenolics by 25–30% in the blueberry cultivar 'Duke'. Similarly, Gündüz and Özbay (2018) found that higher altitude produced fruit that had 17.6% higher total phenolic content, while lower altitude increased the total anthocyanin content by 14.1%. The increase in the content of flavonoids with increasing altitude is comparable to the findings in the research by Dong et al. (2011). By contrast, Bernal et al. (2013) reported that the total amount of flavonoids decreased with increasing altitude, while Rieger et al. (2008) found that, as altitude increased, the content of anthocyanins decreased and the levels of flavonoids increased. Moreover, Guerrero-Chavez et al. (2015) observed that fruits grown at higher altitudes have a lower accumulation of anthocyanins, which is generally reflected in lower antioxidant capacity values, while altitude has no impact on total phenolic content.

Polyphenols are secondary metabolites of great importance as the result of their antioxidant activity, inactivation of lipid free radical chains and prevention of hydroperoxide conversions into reactive oxyradicals. The antioxidant capacity of fruit extracts in the present research was determined by different methods, including total antioxidant capacity, hydroxyl radical scavenging activity, inhibition of lipid peroxidation, and ABTS and DPPH assays. These results are shown in Table 2, as IC50 values.

The results showed high antioxidant potential of fruit extracts of black mulberry, which can be attributed to a high level of flavonoids and anthocyanins, which are considered to be the phenolic compounds with the highest antioxidant activity (Hassimotto et al. 2008). Harborne and Williams (2000) reported that anthocyanins exhibit high antioxidant activities in several oxidation systems, which is related to their ability to inhibit lipid peroxidation and radical scavenging activity. In the present study, the ABTS assay at all altitudes indicated stronger antioxidant activity compared to the DPPH assay. Mean values varied significantly from 52.34 to 54.48 µg/mL (ABTS assay) and from 55.23 to 58.21 µg/mL (DPPH assay). The detected amounts of inhibition of lipid peroxidation ranged from 31.75 to 34.27 µg/mL, while hydroxyl radical scavenging activities were in the range of 41.77–43.12 µg/mL. High antioxidant capacity in black mulberry fruit was reported by Özgen et al. (2009), Ercisli et al. (2010) and Gundogdu et al. (2011). Many studies have shown that environmental differences, including altitude, lead to differences in active ingredient contents and antioxidant activity (Dong et al. 2011; Wang et al. 2014; Liu et al. 2015). The present results indicated a significant effect of altitude on antioxidant activity. The highest antioxidant activity was determined at the lowest-altitude location (187 m) in comparison with the locations at 271 m and 493 m. These results are in agreement with the findings of Guerrero-Chavez et al. (2015), who observed that antioxidant activity in strawberry fruits declined with increasing altitude. By contrast, Mpofu et al. (2006) reported a highly significant effect of location, and that wheat had the highest antioxidant activity at the highest altitude, while Gündüz and Özbay (2018) found that antioxidant capacity is greater at higher altitudes compared to lower altitudes, but this effect of altitude is not very significant. On the other hand, Doumett et al. (2011) observed no altitude-dependent differences in secondary metabolites and radical scavenging activity. In general, Spitaler et al. (2006) recorded that an increase in antioxidant phenolic content corresponds to an increase in the radical scavenging potential of extracts from plants grown at different altitudes.

Interactions between plants and their environment lead to the production of different biologically active substances (secondary metabolites). Many of them show clear antimi-

Table 2 Antioxidant activity in black mulberry fruit

	IC50 (µg/mL)			
	DPPH scavenging activity	Lipid peroxidation inhibitory activity	Hydroxyl radical scavenging activity	ABTS radical scavenging assay
Location 1 (187 m a.s.l.)	55.23 ± 0.85 c	31.75 ± 0.55 c	41.77 ± 0.54 c	52.34 ± 1.11 c
Location 2 (271 m a.s.l.)	56.66 ± 0.14 b	33.83 ± 0.84 b	42.06 ± 0.64 b	53.27 ± 1.03 b
Location 3 (493 m a.s.l.)	58.21 ± 0.65 a	34.27 ± 1.85 a	43.12 ± 1.08 a	54.48 ± 0.76 a
Gallic acid	3.79 ± 0.69	255.43 ± 11.68	59.14 ± 1.10	1.96 ± 0.41
Ascorbic acid	6.05 ± 0.34	>1000	160.55 ± 2.31	10.98 ± 0.95
BHT	15.61 ± 1.26	1.00 ± 0.23	33.92 ± 0.79	7.23 ± 0.87
α-Tocopherol	–	0.48 ± 0.05	–	–

Means followed by different letters within rows are significantly different at $p \leq 0.05$ according to LSD test and ANOVA (F test) results

Table 3 Antimicrobial activity in black mulberry fruit

Microbial strains	MIC values (μg/mL)				
	Location 1 (187 m a.s.l.)	Location 2 (271 m a.s.l.)	Location 3 (493 m a.s.l.)	A[a]	N[b]
Staphylococcus aureus	156.25	78.125	62.60	0.97	/
Klebsiella pneumoniae	156.25	78.125	78.125	0.49	/
Escherichia coli	58.99	19.53	19.53	0.97	/
Proteus vulgaris	78.125	39.1	78.125	0.49	/
Proteus mirabilis	19.53	78.125	78.125	0.49	/
Bacillus subtilis	62.60	78.125	143.24	0.24	/
Candida albicans	78.125	39.1	78.125	/	1.95
Aspergillus niger	39.1	39.1	39.1	/	0.97

[a]Amracin
[b]Nystatin

crobial effects and affect a range of physiological processes in the human body, and provide protection against both free radicals and growth of undesirable microorganisms (Gottschling et al. 2001). Purple and red fruits with a high content of anthocyanins have a significant antibacterial effect (Harborne and Williams 2000). The results on the antimicrobial activity obtained by the dilution method are given in Table 3.

Minimum inhibitory concentrations were determined for eight selected microbial strains. Black mulberry fruits showed antimicrobial activity, with MIC values ranging from 19.53 to 156.25 μg/mL, which is considered to be good antimicrobial activity compared to the standard antibiotics amracin (for bacteria) and nystatin (for fungi). The most sensitive was *Escherichia coli* with an MIC of 19.53 μg/mL at altitudes of 271 m and 493 m, followed by *Aspergillus niger* with an MIC of 39.1 μg/mL at all altitudes and *Candida albicans* with an MIC of 39.1 μg/mL at 271 m. The lowest sensitivity was shown by *Klebsiella pneumoniae* and *Staphylococcus aureus* with the MIC value of 156.25 μg/mL at 187 m altitude. The other Gram-positive and Gram-negative bacteria showed resistance to the tested fruits. Khalid et al. (2011) evaluated the antimicrobial activity of fresh black mulberry juice against various pathogenic bacteria and the results demonstrated antimicrobial activity against both Gram-positive and Gram-negative species. The author reported that high antioxidant and phenolic contents aid in antimicrobial activity. Chen et al. (2018) studied the antibacterial activities of *Morus nigra* fruits extracts against three inflammatory bacteria (*E. coli, P. aeruginosa* and *S. aureus*), and found that the extract strongly inhibited all three strains. Also, Mazimba et al. (2011) determined that five *Morus nigra* fruit extracts showed antibacterial activities against *S. aureus, B. subtilis, Micrococcus flavus, S. faecalis, Salmonella abony* and *P. aeruginosa*, while Yigit et al. (2007) reported strong anticandidal activities of black mulberry fruits against some *Candida* spp. Since polyphenols are potent antimicrobial agents, high antimicrobial activity of fruit extracts can be associated with high total phenol, flavonoid and anthocyanin concentrations in fruit extracts.

Cytotoxic effects of fruit extracts were evaluated using three cell lines: a cell line derived from human rhabdomyosarcoma (RD), a cell line derived from human cervix carcinoma Hep2c (HeLa) and a cell line derived from murine fibroblast (L2OB). The results obtained are shown in Table 4.

Cytotoxic activity was highest in the fruits of black mulberries grown at an altitude of 187 m (location 1) in comparison with location 2 (271 m altitude) and location 3 (493 m altitude). The concentrations of extracts which inhibited 50% of cell growth for Hep2c, RD and L2OB at location 1 were 18.55, 15.32, and 37.17 μg/mL, respectively. *Morus nigra* extracts proved to be potent inhibitors

Table 4 Cytotoxic activity in black mulberry fruit

Cell line	IC50 (μg/mL)			
	Location 1 187 m a.s.l.	Location 2 271 m a.s.l.	Location 3 493 m a.s.l.	*Cis*-DDP[a]
Hep2c cells	18.55 ± 0.33 c	18.95 ± 0.96 b	19.87 ± 0.63 a	0.72 ± 0.64
RD cells	15.32 ± 0.98 c	15.64 ± 0.75 b	16.87 ± 0.36 a	1.40 ± 0.97
L2OB cells	37.17 ± 0.35 c	37.97 ± 0.22 b	38.29 ± 0.48 a	0.94 ± 0.55

Means followed by different letters within rows are significantly different at $p \leq 0.05$ according to LSD test and ANOVA (F test) results
[a]*Cis*-diammine dichloroplatinum

of growth of all three cell lines. According to Çakıroğlu et al. (2017), fruits of *Morus nigra* showed anti-proliferative and apoptotic effects against several human adenocarcinoma cell lines. Also, Huang et al. (2011) reported that anthocyanins from mulberry exhibit activity against gastric cancer, while Jeong et al. (2010) determined that mulberry fruit extracts have a cytotoxic effect on human glioma cells through ROS-dependent mitochondrial pathway and induction of apoptosis. As found by Ahmed et al. (2016), fresh and dried fruit extracts of *Morus nigra* dose- and time-dependently inhibit cellular growth of MCF-7 cells and induce DNA fragmentations and single-strand breaks, and decrease mitotic indexes, with better pharmacological properties in fresh fruit of *Morus nigra*. An antitumor effect of anthocyanins, isolated from berries of *Morus nigra*, on A549 lung carcinoma cells and the cytotoxic activity of mulberry fruit extracts against hepatoma cells (liver cancer) were demonstrated by Chen et al. (2006). Also, in a study by Turan et al. (2017), the anticancer activity of *Morus nigra* fruit extract against PC-3 cells (human prostate cancer) showed that the extract significantly increased the number of necrotic, early apoptotic and late apoptotic cells compared to the untreated control group, and decreased the mitochondrial membrane potential and increased the activities of caspase 3 and 7 in PC-3 cells. In general, the cytotoxic activity of the extract may be the result of a high content of polyphenols, which have various health benefits.

Conclusion

The results of the present research reveal that altitude has a significant effect on the synthesis and accumulation of polyphenols, and a strong influence on antioxidant, antimicrobial and cytotoxic activities in black mulberry fruit, but the effect of altitude on secondary metabolites in the fruit requires further research. Moreover, black mulberries are a rich source of polyphenols and show significant antioxidant, antimicrobial and cytotoxic activities. Therefore, this research is important for assessing the potential effect of altitude on the accumulation and synthesis of secondary metabolites in the fruit, as well as the use of black mulberry fruits as new healthy food ingredients, medical compounds, and pharmaceuticals, which can be highly beneficial for human health.

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Conflict of interest S.M. Paunović, P. Mašković and M. Milinković declare that there are no conflicts of interest.

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