Black Trumpet, *Craterellus cornucopioides* (L.) Pers.: Culinary Mushroom with Angiotensin Converting Enzyme Inhibitory and Cytotoxic Activity

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Nutritional value and chemical composition, including the content of vitamins, fatty acids, 5'-nucleotides and nucleosides and amino acids, as well as biological activities, including antioxidant, angiotensin converting enzyme (ACE) inhibitory and cytotoxic activity of black trumpet (*Craterellus cornucopioides* (L.) Pers.) were tested in vitro. *C. cornucopioides* was low in energy, fat and carbohydrate contents, but rich in dietary fibre, especially β-glucan as well as niacin and α-tocopherol. The content of essential and non-essential free amino acids was 1.49 and 5.48 mg/g dry weight (dw). The nucleosides and 5'-nucleotides were determined at 1.84 and 3.99 mg/g dw, respectively. The share of unsaturated fatty acids (UFAs) was 75.92% with oleic acid as the major UFA. Cyclohexane and dichloromethane extracts expressed significant cytotoxic activity against selected cell lines, human epithelial cervical cancer cells (HeLa), adenocarcinomic human alveolar basal epithelial cells (A549), colorectal cancer cells (LS174) and normal MRC-5 human embryonic lung fibroblast cells (IC_{50} of 78.3–155.6 μg/mL). ACE inhibitory activity of the aqueous extract was strong with an IC_{50} of 0.74 μg/mL. It can be concluded that black trumpet is a good source of nutrients, such as vitamins, dietary fibres, amino acids, nucleotides and fatty acids, which contribute to the overall nutritional value of this fungus with potential for ACE inhibitory activity and use in anti-hypertensive diet.

INTRODUCTION

Black trumpet, *Craterellus cornucopioides* (L.) Pers. is a delicious edible mushroom from the family Cantharelaceae. It is black-brown or almost black mushroom without determined separation of fruiting body between stalk and cap. It looks like a trumpet at the bottom of the tank and slowly extends to the edges. This mushroom grows in groups, in deciduous forests especially on oak and beech [Wheeler & Jordan, 2007]. Primary metabolites (fatty acids, lipids, carbohydrates, amino acids, S'-nucleotides) and secondary metabolites (phenolic compounds and sterols) were previously identified in *C. cornucopioides* [Beluhan & Ranogajec, 2011; Liu et al., 2012; Palaciosetal., 2011]. Considerable amounts of vitamin C, lycopene, β-carotene, α- and γ-tocopherols, and β-glucan were determined as well [Gil-Ramirez et al., 2011; Liu et al., 2012; Vamanu & Nita, 2014; Watanabe et al., 2012]. It was found that the bioactive compounds of *C. cornucopioides* were responsible for several of its biological activities, including: anti-inflammatory, antimutagenic, cytotoxic, hypoglycemic and antioxidant ones [Kosanić et al., 2019; Liu et al., 2012; O’Callaghan et al., 2015; Palacios et al., 2011; Vamanu & Nita, 2014].

High blood pressure and cardiovascular diseases are one of the risk factors for morbidity in both developed and underdeveloped countries. The main risk factors for hypertension that can be influenced are unhealthy diet (excessive salt intake, diet rich in fats and trans fats, low intake of fruits and vegetables), insufficient physical activity and sedentary lifestyle, smoking and alcohol abuse, overweight or obesity [World Health Organization Cardiovascular diseases factsheet, 2021, https://www.who.int/news-room/fact-sheets/detail/cardiovascular-diseases-(cvds)]. According to the recommendations of the European Society of Cardiology and the British Society of Hypertension, therapy of hypertension begins with either angiotensin converting enzyme (ACE)
inhibitors or angiotensin II receptor (AT1) antagonists in patients who have normal or increased plasma renin, thiazide diuretics or calcium antagonists [Williams et al., 2018]. If an adequate antihypertensive effect is not achieved and the drug is well tolerated, an antihypertensive agent from another group is added (thiazide diuretics, Ca2+ antagonists, β-adrenoceptor antagonists, α1-adrenoceptor antagonists) [Williams et al., 2018]. Previous studies have shown significant ACE inhibitory activity of different mushrooms, their extracts and different isolates in vitro and in vivo [Kundaković & Kolundžić, 2013], whereas clinical trials have confirmed the effects of individual mushrooms on lowering blood pressure [Khatun et al., 2007; Lo et al., 2012]. Mushrooms represent a significant source of bioactive peptides and other compounds that show ACE inhibitory activity. A large number of ACE inhibitory peptides have been isolated from edible mushrooms in the last twenty years, e.g. Grifola frondosa [Choi et al., 2001], Mycoleptodonoides aitchisonii [Sakamoto et al., 2001], Tricholoma giganteum [Lee et al., 2004], and Ganoderma lucidum [Wu et al., 2019]. In addition to peptides and oligopeptides, triterpenoids (ganoderic acid F) and D-mannitol of mushrooms have also been shown to elicit an inhibitory effect on ACE activity [Kundaković & Kolundžić, 2013].

Regarding antitumor activity, the effects have been shown for various mushrooms and their isolated compounds on different cell lines. For example, 3β,5α,9α-trihydroxy-ergosta-7,22-dien-6-one isolated from Valvariella volvacea showed significant antitumor activity against human liver cancer cell line (HePG-2) and human gastric cancer cell line (SGC-7901), while ergosterol and ergosterol peroxy expressed significant activity against human prostatic carcinoma cell line (PC-3M) [Chen et al., 2020]. Also, mushroom polysaccharides (e.g., lentinan isolated from Lentinula edodes) have been reported to elicit the antitumor effect via different mechanisms including cancer-preventing activity, immune-enhancing activity and direct tumor inhibition activity [Pandya et al., 2019]. Therefore, guided by the promising findings of previous research, we aimed to evaluate the ACE inhibitory activity of aqueous extracts of wild growing C. corncupioides collected in Bosnia and Herzegovina and to test antitumor activity of its different extracts on several cell lines, in addition to the evaluation of nutritional value, chemical composition and radical scavenging activity.

MATERIAL AND METHODS

Materials and reagents

The following reagents were used: mushroom and yeast β-glucan assay kit K-YBGL and total dietary fibre assay kit K-TDFR obtained from Megazyme Int. (Wicklow, Ireland); ACE Kit-WST obtained from Dojindo Laboratories (Kumamoto, Japan). Analytical grade methanol was obtained from Macron Fine Chemicals (Avantor, Radnor, PA, USA); analytical grade cyclohexane, HPLC grade acetonitrile, methanol and ethanol, as well as sulfuric acid were obtained from Fisher Scientific (Fair Lawn, NJ, USA). Analytical grade dichloromethane, hexane and petroleum ether, tetrabutylammonium bromide (TBAB), 2,2-diphenyl-1-picrylhydrazil radical (DPPH·), Folin-Ciocalteu reagent, as well as analytical standards of vitamins (thiamine hydrochloride (B1), riboflavin (B2), nicotinamide (B3) and pyridoxine hydrochloride (B6)), amino acids and 5'-nucleotides and nucleoside analytical standards, RPMI-1640 medium, fetal bovine serum (FBS), l-glutamine, streptomycin, penicillin, HEPES buffer solution and cis-diaminedichloroplatinum (cis-DDP) were purchased form Sigma-Aldrich (St. Louis, MO, USA). Cholecalciferol (vitamin D3), vitamin A palmitate and dl-α-tocopherol (vitamin E) analytical standards were obtained from Dr. Ehrenstorfer GmbH (Augsburg, Germany); dimethylformamide and triethylamine were obtained from Acros Organics (Geel, Belgium); gallic acid and dimethyl sulfoxide from Carl Roth (Karlsruhe, Germany). Potassium dihydrogen phosphate, sodium bicarbonate, sodium carbonate and sodium phosphate were purchased form Centromer (Stara Pazova, Serbia); and hydrochloric acid from Zorka Pharma (Sabac, Serbia). Fatty acid methyl esters (Supelco 37 Component FAME Mix) and dabsyl chloride solution were obtained from Supelco (Bellefonte, PA, USA). Formic acid was provided by Honeywell (Honeywell International, Inc., Morristown, NJ, USA) and ammonium formate by Agilent Technologies, Inc. (Santa Clara, CA, USA).

Sample collection

Fruiting bodies of the mushroom Craterellus corncupioiides (L.) Pers. (Cantharellaceae family) were collected in Bosnia and Herzegovina, on the northern slopes of mountain Kozara (Kozarska Dubica) in July 2014 (GPS coordinates were: 45°05’25.7”N; 16°51’33.0”E), near the Monastery Moštanica.

Before extraction, the mushroom was dried at room temperature, pulverized in a sample laboratory mill (MRC SM-450, MRC-Laboratory Equipment, Essex, United Kingdom) and stored at 4°C. The voucher specimen was deposited at the Department of Pharmacognosy, Faculty of Pharmacy, University of Belgrade, Republic of Serbia (No 29).

Extract preparation

Extraction was carried out as described previously by Kolundžić et al. [2017] with solvents of different polarity including methanol, cyclohexane and dichloromethane. The dry powdered mushroom (20 g) was weighed and extracted once for two days with each mentioned solvent, at room temperature with a powder to solvent ratio of 1:10 (w/v). The organic solvents were evaporated under low pressure and dried to obtain the black-colored extracts: 0.34 g of cyclohexane extract (CCC), 0.11 g of dichloromethane extract (CCD) and 0.14 g of methanol extract (CCM). Aqueous extract was prepared with 1 g of dried, powdered mushroom mixed with 120 mL of distilled water, heated in a water bath for 30 min at 100°C with occasional shaking. After extraction, water was removed using an Alpha 1–4 LD plus freeze dryer (Martin Christ, Osterode am Harz, Germany) to obtain 0.45 g of dry extract (CCD).

Determination of nutritional value

The dried mushroom was analyzed for the contents of moisture, crude protein, crude fat, total dietary fibre and crude ash. Its nutritional value was determined by using the procedures described by the Association of Official
Analytical Chemists [AOAC, 1995]. Gravimetric method was deployed to determine the moisture and crude ash contents after drying at 105°C and incineration at 550°C, respectively. The crude protein content was determined by the Kjeldahl method using a nitrogen conversion factor of 4.38 for protein calculation. The crude fat content was determined using the Soxhlet extraction with petroleum ether as a solvent, after the treatment with HCl. Total carbohydrate content was calculated as the residual difference after subtracting crude protein, crude ash, moisture, total dietary fibre and crude fat content from 100. The content of total dietary fibre was determined according to the instruction manual of the assay kit (K-TDFR, Megazyme Int.). All results were expressed in g/100 g of dry weight (dw) of mushroom.

**Determination of the content of glucans**

The content of glucans (including α-glucan, β-glucan and total glucan) was determined according to the instruction manual of the mushroom and yeast β-glucan assay kit (K-YBGL) (Megazyme Int.), in dried, powdered mushroom (DM), in mushrooms after thermal treatment (cooking at 100°C for 30 min) (DMAC) and in CCA, CCM, CCC and CCD. Contents of total glucans and α-glucan were determined measuring absorbance at 510 nm on an Evolution 300 UV-Vis spectrophotometer (Thermo Scientific, Madison, WI, USA). The content of β-glucans was calculated indirectly from the difference between the content of total glucans and α-glucan. Results were expressed in g/100 g dw of mushroom or extract.

**Determination of vitamin contents**

The content of thiamine, riboflavin, niacin, pyridoxine, as water-soluble vitamins B$_1$, B$_2$, B$_6$, and B$_9$, respectively, was determined in CCA and CCD, whereas the content of retinol, cholecalciferol and α-tocopherol as fat-soluble vitamins A, D, and E, respectively, was determined in CCC. Before the analysis, methanol and aqueous extracts were diluted with water, whereas cyclohexane extract was diluted with hexane, followed by the filtration of solutions through a 0.45 μm filter. An Agilent 1200 series HPLC with diode array detector (DAD) and a Zorbax Eclipse XDB-C18 (4.6×250 mm, 5 μm) reverse phase column (Agilent), thermostated at 25°C, was used for determinations.

Five microliters of polar extracts, used for the analysis of water-soluble vitamins, were injected into the column. The solvent A consisting of 0.1 M phosphate buffer, pH 2.8 and acetonitrile (90:10, v/v) was used as an eluent for 10 min at the flow rate of 1.0 mL/min. The absorbance for thiamine and niacin was measured at 254 nm, for riboflavin at 268 nm and for pyridoxine at 291 nm. For the analysis of fat-soluble vitamins, the non-polar extract was injected at the volume of 10 μL into the same column. The gradient solvent system, which consisted of mobile phase A and acetonitrile (B), was applied (1% of B for 4.5 min, and then 90% of B until 6 min) with the flow rate of 0.5 mL/min. The absorbances for retinol, cholecalciferol and α-tocopherol determination were measured at 326, 266 and 210 nm, respectively. Identification of vitamins in the samples was performed based on retention times ($t_R$) and UV spectra relative to $t_R$ and UV spectra of the standards. Quantification was performed based on peak areas using baseline construction method. All standard solutions were prepared with a range from 1 to 100 mg/L, except for α-tocopherol with a range of 5–200 mg/L. The content of vitamins was expressed in g/100 g of dry extract.

**Determination of the content of 5'-nucleotides and nucleosides**

For analysing the content of 5'-nucleotides and nucleosides, the samples were prepared according to Beluhan & Ranogajec [2011], with slight modifications. Dried homogenized mushroom weighing 500 mg was mixed with 10 mL of deionized water in a round bottom flask. The suspension was heated on a water bath for 5 min and then stirred with a vortex stirrer for 30 s and cooled to room temperature. The samples were filtered through a 0.45 μm diameter filter before analysis. Stock standard solutions of single 5'-nucleotides (5'-adenosine monophosphate, 5'-AMP; 5'-citidine monophosphate, 5'-CMP; 5'-uridine monophosphate, 5'-UMP; and 5'-guanosine monophosphate, 5'-GMP) and nucleosides (adenosine, cytidine, uridine, guanosine) were prepared at a concentration of 0.5 mg/mL. A series of dilutions (0.25, 0.125, 0.0625, 0.0312 and 0.0156 mg/mL) were prepared from the stock standard solution for the construction of the calibration curves of each of the eight listed substances. All standard solutions were filtered through a 0.45 μm diameter filter. The stock standard solutions were stored at 4°C protected from light until the beginning of the analysis.

Identification and content determination of 5'-nucleotides and nucleosides was performed according to a modified method of Ranogajec et al. [2009] on an Agilent 1100 HPLC system with a binary pump, a G1315B DAD and a Zorbax Eclipse XDB-C18 column (4.6×250 mm, 5 μm). Twenty microliter of the test sample and standard solutions were injected. The solvent system was composed of two mobile phases: 0.1 M KH$_2$PO$_4$ and 4 mM tetrabutylammonium bromide, pH 6 (A) and a mixture of mobile phase A with methanol (70:30, v/v), pH 7.2 (B). Elution started at the flow rate of 1.2 mL/min with 5% B until 9 min, 25% B at 15 min, 90% B at 17.5 min, 100% B at 19 min and 5% B at 24 min. The absorbance was recorded at 254 nm. Identification of 5'-nucleotides and nucleosides in the sample was performed based on $t_R$ and UV spectra relative to $t_R$ and UV spectra of the standards. Quantification was performed based on peak areas using baseline construction method. The content of 5'-nucleotides and nucleosides was expressed in mg/g dw of mushroom.

**Analysis of free amino acid composition and umami potential**

Samples of dried mushroom were prepared as described above for the analysis of the contents of 5'-nucleotides and nucleosides. An amino acid standard solution containing l-alanine, l-arginine, l-glutamic acid, l-cystine, l-aspartic acid, l-glycine, l-histidine, l-isoleucine, l-leucine, l-lysine, l-methionine, l-phenylalanine, l-proline, l-serine, l-threonine, l-tyrosine and l-valine was used to prepare stock standard solutions and their dilutions (250, 125, 50, 25, 12.5, 6.25, 3.125, 1.5625 and 0.0678 μM).

Before the analysis, derivatization of the standards and the amino acids of the test sample was performed...
according to Ribeiro et al. [2008]. Briefly, 20 μL of both the test solution and standard solutions was mixed with 180 μL of a reaction buffer (0.1 M NaHCO₃, pH 8.6), stirred, and 200 μL of dabsyl chloride solution (12.4 mM) was added. The vials were stirred and heated to 70°C for 15 min. The reaction was ended by cooling on ice for 5 min. Then, 400 μL of a dilution buffer was added, containing 50 mL of acetonitrile, 25 mL of ethanol and 25 mL of an elution buffer (9 mM sodium phosphate, 4% dimethylformamide, 0.15% triethylamine, pH 6.5). The mixture was centrifuged for 5 min at 1,700×g. The supernatant was filtered and stored at −20°C until the analysis.

The analysis of amino acids was performed using the LC-MS-ESI system (Agilent 1260/6130) equipped in an Agilent Zorbax SB-Aq column (3.0×150 mm, 3.5 μm) thermostated at 25°C. The volume of injection was 3 μL. The mobile phase system was composed of two solvents: 0.1% (v/v) ammonium formate in water (A) and 0.1% (v/v) formic acid in acetonitrile (B). Elution started at the flow rate of 0.3 mL/min with 15% B until 3 min, 85% B at 63 min and 15% B at 74 min. Detection wavelengths were at 250 nm. Electrospay ionization (ESI) mass spectra were recorded in the positive ion mode in a range of 50–600 m/z; fragmentator voltage of 250, nebulizer gas of He, used at the flow rate of 1.2 mL/min at 350°C, under pressure of 40 psi and capillary voltage of 3500 V. Individual amino acids were identified in the mixture based on their tₚ and mass spectra in relation to the tₛ and mass spectra of the corresponding standards, analysed under the same chromatographic conditions. The amino acid contents were calculated based on the peak area using baseline construction method. The content of individual amino acids was expressed in mg/g dw of mushroom. Also, to evaluate the umami potential of our tested mushroom sample, the equivalent umami concentration (EUC) value was calculated according to the formula given by Yamaguchi et al. [1971]. The EUC value of 100% indicates that the umami intensity of fruit bodies or mycelia per 1 g dry weight is equivalent to the umami intensity given by 1 g of monosodium glutamate (MSG).

**Determination of fatty acid composition**

Fatty acid composition of CCC was analyzed according to the method described by Ušjak et al. [2019] using an Agilent 6890N gas chromatography (GC) system equipped with a split/splitless injector (260°C), a flame ionization detector (FID) and a capillary column (Agilent J&W HP-88, 100 m × 0.25 mm, 0.20 μm film thickness), coupled with an Agilent 5975C mass spectrometry (MS) detector operating in the electron ionization mode at 70 eV. Saponification was achieved with 50% KOH at 90°C for 1 h. Petroleum ether was used to separate unsaponifiable fractions. Fatty acid methyl esters (FAME) were obtained by esterification of fatty acids with 98% H₂SO₄/anhydrous MeOH. The carrier gas was He, used at the flow rate of 1.2 mL/min. The oven temperature was initially held at 140°C for 5 min, then increased linearly from 140°C to 240°C at 4°C/min, and finally held at 240°C for 10 min. The FID and MS transfer line temperatures were 260°C and 250°C, respectively. Split ratio was 1:25 and the injected volume was 1 μL of 1% solution of the FAME in dichloromethane.

The identification of the FAME was based on the comparison of their tₚ and mass spectra to those of the representative standards ran under the same chromatographic conditions and to those from the NIST/NBS 05 and Wiley (8th edition) libraries, and the literature. Relative percentages of the compounds were calculated based on the peak areas.

**Determination of total phenolic content**

The content of total phenolics in CCM and CCA was determined spectrophotometrically, as described previously by Kolundžić et al. [2017]. To this end, 100 μL of methanol solution of CCM (11 mg/mL) and CCA (12 mg/mL) was mixed with 750 μL of the diluted Folin-Ciocalteu reagent (1:1, v/v, in distilled water). After 5 min, 750 μL of 60 g/L Na₂CO₃ solution was added to each sample. Firstly, the mixtures were shaken and then left for 90 min in the dark at room temperature. Absorbance was measured at 725 nm using UV-Vis spectrophotometer Evolution 300 (Thermo Scientific). As a blank, 100 μL of the solvent was used. Series of gallic acid dilutions (1–10 mg/mL) were reacted in parallel, the absorbance was measured and the calibration curve was constructed. The content of total phenolics was expressed as mg of the gallic acid equivalent (GA)/g of dry extract.

**Determination of DPPH radical scavenging activity**

The ability of CCM and CCA to scavenge free radicals was measured in a DPPH test [Kolundžić et al., 2017]. Briefly, six volumes (10, 30, 50, 70, 90, 100 μL) of stock methanol solutions of extracts (CCM 60 mg/mL; CCA 16 mg/mL) were mixed with methanol, so that the total volume became 2 mL, and 0.5 mL of 0.5 mM DPPH radical solution in methanol was added. The concentration range was 240, 720, 1200, 1680, 2160 and 2400 μg/mL for CCM and 64, 192, 320, 448, 576 and 640 μg/mL for CCA. The mixtures were left in the dark at room temperature for 30 min. The absorbance of the solution was measured at 517 nm using an Evolution 300 UV-Vis spectrophotometer (Thermo Scientific) and the percent of inhibition of DPPH radical was calculated. The results were expressed as a IC₅₀ value, i.e. concentration of the extract that reduced 50% of the DPPH radicals.

**Determination of ACE inhibitory activity**

The ACE Kit-WST (Dojin Laboratories) was used to determine ACE inhibiting activity of CCA. In the assay, 3-hydroxybutyric acid (3HB), which is released from 3-hydroxybutyryl-Gly-Gly-Gly (3HB-GGG), was enzymatically detected. The concentrations ranging between 0.1 and 20 mg/mL of the aqueous extract were used and the procedure of the manufacturer was completely followed. The experiment was done in the 96-well microtiter plate. The absorbance of the solutions was measured on an EL×800 absorbance microplate reader (BioTek Instruments, Winooski, VT, USA) at 450 nm and the extract concentration needed to inhibit 50% of ACE activity (IC₅₀) was calculated.

**Cytotoxicity assay**

Treatment of cell lines and determination of cell survival rate were done according to the procedure described by Kolundžić et al. [2017]. Human epithelial cervical cancer
cells HeLa, adenocarcinomic human alveolar basal epithelial cells AS49, colorectal cancer cells LS174 and normal MRC-5 human embryonic lung fibroblast cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). All cancer cell lines were maintained in the recommended RPMI-1640 medium supplemented with 10% heat-inactivated (56°C) FBS, l-glutamine (3 mM), streptomycin (100 mg/mL), penicillin (100 IU/mL), and 25 mM HEPES buffer and adjusted to pH 7.2 by bicarbonate solution. Cells were grown in a humidified atmosphere of 95% air and 5% CO₂ at 37°C. Stock solutions (100 mg/mL) of four extracts were made in dimethyl sulfoxide and dissolved in corresponding medium to the required working concentrations. Final concentrations achieved in the treated wells were 12.5, 25, 50, 100 and 200 μg/mL. The effects on cancer cell survival were determined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT) test, 72 h upon extract addition, as described previously [Kolundžić et al., 2017]. 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 (Thermo Labsystem Multiskan EX, Thermo Fisher scientific, Waltham, MA, USA) at 570 nm. IC₅₀ was defined as the concentration of an agent inhibiting cell survival by 50%, compared with a vehicle-treated control. Cisplatin (cis-DDP) was used as a positive control.

Statistical analysis

All measurements were done in three analytical repetitions. Data are presented as mean ± standard deviation (SD). Statistical analyses were conducted using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). Data were statistically analysed using Student's t-test (for two groups) or one-way analysis of variance (ANOVA) with post-hoc Tukey’s test (for three or more groups), where appropriate. Differences were considered statistically significant at p<0.05.

RESULTS AND DISCUSSION

Nutritional value, contents of total dietary fibre and glucans

Fruiting bodies of C. cornucopioides were low in crude fat (5.02±0.16 g/100 g dw), total carbohydrates (11.97±0.87 g/100 g dw) and crude proteins (11.51±0.16 g/100 g dw), which resulted in a low energy value (248.03±9.84 kcal/100 g dw; 1037.63±31.00 kJ/100 g dw). The moisture content was 6.84±0.10 g/100 g dw, and crude ash was 10.20±0.12 g/100 g dw. Results obtained in this study are in correlation with previous results in terms of the contents of crude ash and crude fat, but the protein content was far lower than 47.21%, 69.45% and 50.10% reported by Beluhan & Ranogajec [2011], Barros et al. [2008] and Colak et al. [2009], respectively. C. cornucopioides was rich in total dietary fibre (54.46±0.90 g/100 g dw). β-Glucan content in our sample (dried mushroom) was 15.65 g/100 g dw, which accounted for approximately 98% of total glucans (Table 1) and one third of the total dietary fibre. A similar result (16.20 g/100 g dw) has been reported for black trumpet growing in Poland of [Mironczuk-Chodakowska et al., 2017]. Cooking has been reported to influence the proximate glucan content in mushrooms [Kolundžić et al., 2017].

| Table 1. The content of total, α- and β-glucan (g/100 g dw) in dried Craterellus cornucopioides before and after cooking and in extracts of mushrooms. |
|--------------------------------------------------|-------------------|-------------------|
| | Total Glucan | α-Glucan | β-Glucan |
| Dried mushroom | 15.96±0.07a | 0.31±0.01b | 15.65±0.06b |
| Dried mushroom after cooking | 1.53±0.02d | 0.08±0.01e | 1.44±0.03f |
| CCA | 2.54±0.10f | 0.15±0.01g | 2.39±0.10g |
| CCM | 1.61±0.04h | 0.13±0.01i | 1.47±0.04i |

Results are expressed as mean ± standard deviation (n=3). Values with different superscript letters in column differ significantly at p<0.05. CCA, C. cornucopioides extracts (A, aqueous; M, methanol); dw, dry weight.

In the present study, the content of total glucans decreased significantly (p<0.05) after cooking to 1.53 g/100 g dw, indicating approximately 90% reduction (Table 1). The content of glucans was also determined in extracts. The presence of glucans was detected in polar (aqueous and methanol) but not in non-polar extracts (cyclohexane and dichloromethane). The significantly (p<0.05) highest content of total glucans, α- and β-glucan was detected in dried mushrooms compared to extracts. In turn, CCA had a significantly (p<0.05) higher content of all three glucan types (total glucans, α- and β-glucan) compared to CCm, except for α-glucan in CCM. Therefore, it can be concluded that glucan contents in mushrooms depend not only on the cooking process, but also on the extraction method as well as the choice of solvent used for extraction.

Vitamins

The contents of water-soluble vitamins B₁, B₂, B₃ and B₆ were determined in polar extracts (CCA and CCM), while those of fat-soluble vitamins A, D₃ and E were determined in the non-polar extract (CCm) (Table 2). The most abundant water-soluble vitamin was B₁, niacin with the content of 249.0 µg/100 g dw CCA and 367.0 mg/100 g dw CCM, followed by vitamin B₂, riboflavin, vitamin B₃, thiamine and vitamin B₆, pyridoxine. ANOVA showed a significantly higher vitamin B₁ content in CCA compared to CCM (p<0.05), and significantly higher contents of vitamin B₂ and B₃ in CCM compared to CCA (p<0.05). So far, the content of B-group vitamins in C. cornucopioides has not been determined extensively. Çağlarımak [2011] reported thiamine, riboflavin, pyridoxine and niacin contents of 0.17–0.63, 0.26–0.90, 0.14–0.56 and 6.38–8.37 mg/100 g dw, respectively, in dried cultivated shiitake, white and brown button mushrooms and oyster mushroom.

α-Tocopherol is the most active form of vitamin E in humans and as a free radical scavenger it protects our bodies against degenerative diseases [Tucker et al., 2005]. α-Tocopherol was found as the most abundant fat-soluble vitamin in the cyclohexane extract of C. cornucopioides, with the content of 2816 mg/100 g dry extract corresponding to 47.90 mg/100 g dw of mushroom. The content of α-tocopherol previously reported in C. cornucopioides was 0.115 mg/100 g dw [Liu et al., 2012], which is much lower than the value reported in our study. In turn, contents of total tocopherols have
been determined in mushrooms of Boletus species, revealing 2533 μg/100 g dw in B. reticulatus [Heleno et al., 2011].

Group-D vitamins occurred in two forms: ergocalciferol – vitamin D₃ being the major form of vitamin D found in edible mushrooms, and cholecalciferol – vitamin D₃ found in lesser amounts [Cardwell et al., 2018]. Also, ergosterol (one vitamin D precursor) is commonly found in fungi [Venditti et al., 2017]. Considerable amount of vitamin D₃ has been found in the cyclohexane extract of C. cornucopioides, i.e. 89.3 mg/100 g dry extract which corresponded to 1.52 mg/100 g dw of mushroom. There is no literature data that support this finding. So far, only vitamin D₃ has been detected in the wild growing species of the genus Cantharellaceae, i.e. at 10.70 mg/100 g dw in Cantharellus cibarius and 21.10 mg/100 g dw in C. tubaeformis [Teichmann et al., 2007]. In the case of vitamin A, it was detected in the CCC at 16.1 mg/100 g dw (0.27 mg/100 g dw of mushroom), and here likewise, there is no literature data to compare this value.

5'-Mononucleotides and nucleosides

Nucleosides, being precursor molecules for DNA and RNA synthesis, are vital for cellular function. Also, both 5'-nucleotides and nucleosides were reported to exert various positive impacts on brain function, immune system, fatty acid metabolism, and gastrointestinal tract [Phan et al., 2018]. The most common nucleotides found in mushrooms are 5'-adenosine monophosphate (5'-AMP), 5'-cytidine monophosphate (5'-CMP), 5'-guanosine monophosphate (5'-GMP), 5'-inosine monophosphate (5'-IMP), 5'-uridine monophosphate (5'-UMP) and 5'-xanthosine monophosphate (5'-XMP) [Phan et al., 2018].

The total content of 5'-nucleotides in dried body of C. cornucopioides was 3.99 mg/g dw, while the content of nucleosides was 1.84 mg/g dw. The 5'-nucleotide content could be ordered as follows: 5'-UMP > 5'-AMP > 5'-GMP > 5'-CMP (1.55±0.03, 0.87±0.47, 0.83±0.15 and 0.74±0.13 mg/g dw, respectively). In turn, the content of nucleosides decreased in the following order: uridine > adenosine = guanosine > cytidine (0.50±0.01, 0.47±0.07, 0.47±0.03 and 0.40±0.04 mg/g dw, respectively). The findings obtained from this research are different than the results published before. Ranogajec et al. [2009] reported lower contents of the same 5'-nucleosides and nucleotides in C. cornucopioides sample, i.e. 0.30 and 0.91 mg/g dw, respectively, while the content of 5'-nucleotides in C. cornucopioides, reported later by the same research group, was much higher, reaching 10.48 mg/g dw [Beluhan & Ranogajec, 2011]. These differences in the results are due to the influence of different factors on 5'-nucleotide content, such as the use of different parts of the fungus, the stage of fungal development, and collection time, as shown in the example of Tricholoma matsutake where the contents of 5'-AMP and 5'-GMP were higher in its pileus than stalk [Cho et al., 2010].

5'-Nucleotides, such as 5'-GMP, 5'-IMP, 5'-XMP and 5'-AMP, in synergy with glutamic and aspartic acid contribute to the specific umami taste of mushrooms, which is the taste induced by monosodium glutamate (MSG) [Phat et al., 2016]. Among these compounds, 5'-GMP is the one responsible for the meaty taste of mushrooms, and it is a stronger flavor enhancer than MSG [Beluhan & Ranogajec, 2011]. The umami intensity of the mentioned compounds is discussed below in the Free amino acids section.

Free amino acids

The contents of individual free amino acids in dried C. cornucopioides are shown in Table 3. All amino acids, except histidine and cystine, were identified in the sample. The total amino acid content was 6.97 mg/g dw. According to the literature, the content of free amino acids in mushrooms is low and varies from 7.14 mg/g to 12.3 mg/g in dried edible mushrooms, with glutamic acid (21.7–23.7%) and alanine (17.7–17.9%) found as major ones [Cheung, 2010]. The content of amino acids in C. cornucopioides has been previously examined, where higher values were obtained for amino acids (67.48 mg/g dw), with glutamic acid as the most major amino acid [Beluhan & Ranogajec, 2011].

The content of essential amino acids in C. cornucopioides was 1.49 mg/g dw, while non-essential amino acids were present in a higher content of 5.48 mg/g dw (Table 3). The calculated ratio between essential and non-essential amino acids was 0.27, which was below the value of 0.6 recommended by FAO/WHO [Wang et al., 2014].

Another classification of amino acids can be made according to their characteristic taste [Beluhan & Ranogajec, 2011]. Among the C. cornucopioides amino acids were those with sweet taste, including alanine, glycine, serine and threonine; bitter taste, including arginine, isoleucine, leucine, methionine, phenylalanine and valine; tasteless, including lysine and tyrosine; and monosodium glutamate-like (MSG-like) taste, including glutamic and aspartic acids (Table 3). The predominant amino acids were those with bitter taste (3.14 mg/g dw) followed by MSG-like tasting (1.83 mg/g dw) and tasteless (1.00 mg/g dw). The low content of sweet-tasting amino acids (0.68 mg/g dw) was not surprising and it was in accordance with the low content of carbohydrates. Nevertheless, it has been found that sweet and MSG-like amino acids are so-called taste-active amino acids in mushrooms, unlike the bitter-testing ones, and so bitter taste might be suppressed by the sweet components [Mau, 2005].
As mentioned before, the synergistic effect of umami 5'-nucleotides (5'-AMP, 5'-IMP, 5'-GMP, and 5'-XMP) and umami amino acids (glutamic and aspartic acid) may enhance the umami taste of mushrooms. Hence, the EUC value was calculated to evaluate the umami potential of the mushroom sample. The EUC values are grouped into four levels: >1000% (>10 g MSG/g dw); 100–1000% (1–10 g MSG/g dw); 10–100% (0.1–1 g MSG/g dw) and 10% (<0.1 g MSG/g dw) [Mau, 2005]. The EUC value for the tested C. cornucopioides was 36.20%, which classifies it as the mushroom with EUC values at the second and third levels. According to Mau [2005], mush-rooms with EUC values at the second and third levels are those that do not contribute to umami taste but are still used for culinary purposes for their flavor, consistency and texture.

Fatty acids

The fatty acid composition of the cyclohexane extract of C. cornucopioides is shown in Table 4. Saturated fatty acids (SFAs), monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) have been identified and quantified. The most abundant group of fatty acids were MUFAs (61.44%), followed by SFAs (24.08%) and PUFAs (14.48%). This is considered to be a positive ratio since the SFAs are recognized to be associated with cardiovascular disorders and atherosclerosis, while the high intake of PUFAs and MUFAs is considered to prevent these disorders [Silva Figueiredo et al., 2017]. The obtained data are in accordance with findings from previously conducted studies where MUFAs were also detected as the most represented class of fatty acids in C. cornucopioides (59.85%), followed by PUFAs (23.79%) and SFAs (16.36%) [Barros et al., 2008].

The main fatty acid in the tested mushroom was oleic acid (Table 4). This monounsaturated but not an essential fatty acid is effective in lowering cholesterol level, which affects high blood pressure [Abugri et al., 2016]. Essential fatty acids include linoleic acid (C18:2n6c) and α-linolenic acid (C18:3n3c). Since the human organism is not able to synthesize these fatty acids, they must be provided with diet. In our study, only linoleic acid was found in C. cornucopioides with a relative content of 10.85% (Table 4). In turn, stearic acid was the most abundant (12.43%) among the identified saturated fatty acids. However, it has no impact on increasing serum low density lipoprotein (LDL) cholesterol level, due to its rapid conversion into oleic acid in the organism [Grundy, 2013]. Nevertheless, there was a higher content of unsaturated than saturated fatty acids in the tested C. cornucopioides, which can be classified as a healthy food.

Total phenolic content and DPPH radical scavenging activity

Phenolic compounds are very good antioxidants and possess very good radical scavenging ability [Zhao et al., 2014]. The content of total phenolics in the methanol and aqueous extracts of C. cornucopioides is shown in Table 5. The methanol extract contained almost twice less total phenolics than the

### TABLE 3. Free amino acid content in dried Craterellus cornucopioides and taste of amino acids.

<table>
<thead>
<tr>
<th>Type of amino acid</th>
<th>Amino acid</th>
<th>Taste*</th>
<th>Content (mg/g dw)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Essential amino acid</td>
<td>Histidine</td>
<td>Bitter</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Lysine</td>
<td>Tastless</td>
<td>0.38±0.02</td>
</tr>
<tr>
<td></td>
<td>Threonine</td>
<td>Sweet</td>
<td>0.21±0.01</td>
</tr>
<tr>
<td></td>
<td>Methionine</td>
<td>Bitter</td>
<td>0.01±0.00</td>
</tr>
<tr>
<td></td>
<td>Valine</td>
<td>Bitter</td>
<td>0.04±0.01</td>
</tr>
<tr>
<td></td>
<td>Leucine/isoleucine</td>
<td>Bitter</td>
<td>0.68±0.04</td>
</tr>
<tr>
<td></td>
<td>Phenylalanine</td>
<td>Bitter</td>
<td>0.17±0.05</td>
</tr>
<tr>
<td>Non-essential amino acid</td>
<td>Cystine</td>
<td>/</td>
<td>nd</td>
</tr>
<tr>
<td></td>
<td>Arginine</td>
<td>Bitter</td>
<td>2.24±0.01</td>
</tr>
<tr>
<td></td>
<td>Serine</td>
<td>Sweet</td>
<td>0.15±0.06</td>
</tr>
<tr>
<td></td>
<td>Aspartic acid</td>
<td>MSG-like</td>
<td>0.43±0.11</td>
</tr>
<tr>
<td></td>
<td>Glutamic acid</td>
<td>MSG-like</td>
<td>1.40±0.10</td>
</tr>
<tr>
<td></td>
<td>Glycine</td>
<td>Sweet</td>
<td>0.22±0.03</td>
</tr>
<tr>
<td></td>
<td>Alanine</td>
<td>Sweet</td>
<td>0.10±0.03</td>
</tr>
<tr>
<td></td>
<td>Tyrosine</td>
<td>Tasteless</td>
<td>0.62±0.01</td>
</tr>
<tr>
<td></td>
<td>Proline</td>
<td>/</td>
<td>0.32±0.01</td>
</tr>
</tbody>
</table>

*according Beluhan & Ranogajec [2011]. Results are expressed as mean ± standard deviation (n=3). MSG-like, monosodium glutamate-like; nd, not detected; dw, dry weight.

### TABLE 4. Fatty acid composition (% total fatty acids) of Craterellus cornucopioides cyclohexane extract.

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>Content</th>
</tr>
</thead>
<tbody>
<tr>
<td>C 15:0</td>
<td>0.33±0.05</td>
</tr>
<tr>
<td>C 16:0</td>
<td>9.96±0.25</td>
</tr>
<tr>
<td>C 16:1</td>
<td>0.34±0.20</td>
</tr>
<tr>
<td>C 17:0</td>
<td>0.19±0.01</td>
</tr>
<tr>
<td>C 18:0</td>
<td>12.43±0.37</td>
</tr>
<tr>
<td>C 18:1n9c</td>
<td>60.83±1.46</td>
</tr>
<tr>
<td>C 18:2n6c</td>
<td>10.85±0.10</td>
</tr>
<tr>
<td>C 20:0</td>
<td>0.41±0.03</td>
</tr>
<tr>
<td>C 20:5n3</td>
<td>0.70±0.03</td>
</tr>
<tr>
<td>C 22:0</td>
<td>0.36±0.01</td>
</tr>
<tr>
<td>C 22:6n3</td>
<td>2.93±0.21</td>
</tr>
<tr>
<td>C 24:0</td>
<td>0.41±0.08</td>
</tr>
<tr>
<td>C 24:1n15c</td>
<td>0.26±0.06</td>
</tr>
<tr>
<td>SFAs</td>
<td>24.08±0.79</td>
</tr>
<tr>
<td>MUFAs</td>
<td>61.44±1.73</td>
</tr>
<tr>
<td>PUFAs</td>
<td>14.48±0.34</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation (n=3). SFAs, saturated fatty acids; MUFAs, monounsaturated fatty acids; PUFAs, polyunsaturated fatty acids.
a aqueous extract and thus exhibited lower DPPH radical scavenging activity (IC$_{50}$ of 887 μg/mL for CCM and 473 μg/mL for CCA). Previous results of Liu et al. [2012] have shown significant, but lower than the results in this study, DPPH radical scavenging activity of C. cornucopioides extracts with the IC$_{50}$ value of 40.30 mg/mL for ethanol extract and 26.37 mg/mL for aqueous extract and with quercetin as a major compound in the ethanol extract. Vamunu & Nita [2014] have used a different extraction method with a fluidized bed extractor, but the extract produced also had a lower total phenolic content (88.4 mg GA/100 g of extract) than in this study, whereas DPPH radical scavenging activity was about 60% for the extract concentration of 1 mg/mL. Different extraction procedure leads to the extraction of other compounds with antioxidative power as ascorbic acid, lycopene, β-carotene and tocopherols [Vamunu & Nita, 2014], but in our study, lipophilic compounds could accumulate in non-polar cyclohexane extract which was not suitable for antioxidant activity estimation using the DPPH method.

ACE inhibitory activity

The results indicated dose-dependent ACE inhibitory activity of the C. cornucopioides aqueous extract with an IC$_{50}$ of 0.74 mg/mL (Table 5). According to the manufacturer of the ACE kit (Dojindo Laboratories), IC$_{50}$ of captopril – well-known ACE inhibitor – was 2.14 nM (0.465 ng/mL), in the same test [https://dojindo.com/product/ace-kit-wst-a502/]. The results of Kolundžić et al. [2017] study with Cantharellus cibarius from the same family Cantharellaceae showed 68% ACE inhibitory activity at a dose of 1.25 mg/mL. In turn, Jang et al. [2011] isolated and characterized two ACE inhibitors with IC$_{50}$ values of 0.46 and 1.14 mg/mL from fruiting bodies of Pleurotus cornucopiae, and demonstrated an antihypertensive effect in vivo of an aqueous extract of the same fungus. Aqueous and ethanolic extracts of haesongi mushroom (Hypsizigus marmoreus) showed dose-dependent ACE inhibitory activity in vitro (aqueous extract had strong activity: 93.34% at a concentration of 40 mg/mL) [Jung et al., 2009]. Lee et al. [2004] showed ACE inhibitory activity of an aqueous extract of T. giganteum with an IC$_{50}$ of 0.31 mg/mL and of an isolated peptide consisting of proline, glutamic acid and glycine (IC$_{50}$ of 0.04 mg/mL), as well as different ACE inhibitory activities for aqueous, ethanolic and methanolic extracts of several mushrooms: Pleurotus sajor-caju, P. ostreatus, Flammulina velutipes, T. giganteum, Agaricus bisporus, Poria cocos, Grifola umbellata, and Lentinus edodes, but the best activity was shown for the aqueous extract of T. giganteum.

Cytotoxic activity

The cytotoxic effect of four C. cornucopioides extracts was tested against human epithelial carcinoma cells, human lung carcinoma cells, human colon carcinoma cells, as well as on normal cell line. The respective results are presented in Table 6. From the obtained data, it can be concluded that all four extracts of C. cornucopioides expressed moderate cytotoxic activity against the tested cell lines. Non-polar extracts (cyclohexane and dichloromethane) were significantly (p<0.05) more active against the tested cell lines, including normal ones. The highest activity was observed in the cyclohexane extract against human epithelial carcinoma cells (IC$_{50}$ of 78.3 μg/mL). Polar extracts (aqueous and methanol) showed almost no activity against the tested cell lines. The anti-tumor effect of some non-polar compounds has been confirmed before, including fatty acids [Sagar et al., 1992]. Linoleic acid exhibited antimicrobial activity and cytotoxic activity against the HeLa tumor cell line. This fatty acid found as abundant fatty acid in the cyclohexane extract (Table 4) could be responsible for its high cytotoxic activity. Seven illudin sesquiterpenoids, i.e. craterellin A, B and C, illudin F, M, T and illudalenol, and one gymnomitrine sesquiterpenoid, gymnomitrin-3-en-10β,15-diol, have been isolated by Guo et al. [2017a] from an ethyl acetate extract of C. cornucopioides as colorless oils and tested against several cancer cell lines (breast cancer SK-BR-3, hepatocellular carcinoma SMMC-7721, human myeloid leukemia HL-60, pancreatic cancer Panc-1 and lung cancer A-S49). Only craterellin C (sesquiterpenoid) isolated from

<table>
<thead>
<tr>
<th>Total phenolic content (mg GA/g extract)</th>
<th>DPPH scavenging activity (IC$_{50}$ μg/mL)</th>
<th>ACE inhibitory activity (IC$_{50}$ mg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CCA</td>
<td>16.96±0.22 $^a$</td>
<td>473±5 $^b$</td>
</tr>
<tr>
<td>CCM</td>
<td>8.95±0.06 $^b$</td>
<td>887±15 $^c$</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± standard deviation (n=3). Values with different superscript letters in column differ significantly at p<0.05. CC, C. cornucopioides extracts (A, aqueous; M, methanol; GA, gallic acid equivalent).
the ethyl acetate extract of black trumpet, expressed moderate cytotoxicity against A-549 (21.0 μM). Craterellin D and E, as well as menthane monoterpenes, 4-hydroxy-4-isopropenyl-cyclohexanemethanol acetate, have also been isolated from the ethyl acetate extract of C. cornucopioides, and tested against the same cell cultures, but none of the compounds exhibited cytotoxic activity [Guo et al., 2017b]. In turn, methanolic extracts of twenty nine mushroom species, including C. cornucopioides, were tested by MTT assay against human lung adenocarcinoma A549 [Vasdekis et al., 2018]. Black trumpet extract was among the extracts with the most prominent activity with IC₅₀ <1 mg/mL. Piceatannol, the natural stilbene with antioxidant, anticancer and anti-inflammatory activities, was identified in all tested extracts with promising cytotoxic activity. Similar research has recently been conducted by Kosanić et al. [2019]. This research group tested the cytotoxic potential of an acetone extract of C. cornucopioides against the same cell lines as in our research. The tested extract showed a moderate effect, and HeLa were the most sensitive. These authors concluded that this effect might have been due to the presence of polyphenols. The non-polar extract analyzed in our study (CCC) was also the most active against HeLa cell line.

CONCLUSION

Based on the results of this study, it can be concluded that black trumpet was a good source of nutrients, suitable for daily diet, primarily due to its low fat content and high content of dietary fibre, especially β-glucan. The nutritional potential of this fungus was also ascribed to the presence fatty acids, especially oleic acid and linoleic acid, which would be very important for people with hypertension or metabolic syndrome. The most promising result was the inhibitory effect of the aqueous extract on ACE. This was the first time that ACE inhibitory activity of black trumpet extract was tested. Concerning cytotoxicity, the cyclohexane extract, which was rich in fatty acids and vitamin E, exhibited the greatest potential.

In terms of the content of other nutrients and bioactive compounds, this mushroom was rich in vitamins and, to the best of our knowledge, this is the first time that their content was determined in extracts of different polarities, according to the solubility of the vitamins themselves, and not to the best of our knowledge, this is the first time that their content was determined in extracts of different polarities, according to the solubility of the vitamins themselves, and not in fresh mushrooms. The content of amino acids, nucleotides and fatty acids, was obviously lower than in previous studies, but it was still correlated with the overall nutritional potential of this fungus. As expected, the content of total phenolics was also low, which resulted in poor antioxidant activity. Black trumpet should be analyzed in more detail for identification and isolation of specific compounds that contribute to the ACE inhibitory and cytotoxic activity.

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CONFLICT OF INTERESTS

The authors confirm that this article content has no conflict of interest.

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