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Antioxidant and antiproliferative potential of fruiting bodies of wild-growing mushroom *Boletus edulis* (Bull.) collected in Western Serbia

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SHORT TITLE: Bioactivity potential of *Boletus edulis*

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ABSTRACT:*Boletus edulis* is considered to be one among the most known and most delicious mushroom in the world which is commonly consumed and eaten in soups, pasta or risotto. Considering its diverse popularity and use, the aim of this work was to study bioactivity of crude aqueous and ethanolic extracts of *B. edulis*prepared from cap and stipe (BecAq, BesAq, BecEtOH, BesEtOH) of wild-growing sporocarps collected from Prijepolje region (Western Serbia). The bioactivity screening included antioxidant (DPPH•, NO•, SO•, OH• and FRAP) and antiproliferative (human breast MCF-7 cancer cell-line; MTT)assays. In addition, all extracts were primarily characterized by UV-VIS spectrophotometry in order to determine total phenolic and flavonoid contents. The highest anti-DPPH and anti-OH radical activity were observed in BecAq (IC_{50}= 50.97 μg/ml and IC_{50}= 2.05 μg/ml) while the highest anti-NO radical activity was observed in BesAq (IC_{50}= 10.74 μg/ml). The ethanolic extract obtained from the mushroom stipe showed higher anti-SO radical activity (IC_{50}= 9.84 μg/ml) and ferric reducing antioxidant power (22.14mg AAE/g d.w.) compared to aqueous extracts. Total phenolic(TP) content for all extracts was similar but total flavonoid (TF) content was significantly higher in BecAq (4.5 mg QE/g d.w.). All crude extracts showed activity against MCF-7 cell line,with BesEtOH (IC_{50} 56 μg/ml) being the most potent. This is the first report on the antiproliferative effects of crude aqueous and ethanolic extracts prepared from cap and stipe of wild-growing sporocarps of *B. edulis* on human breast MCF-7 cancer cell-line.

KEY WORDS: *Boletus edulis*, fruiting body, phenolic compounds, antiradical and antiproliferative activity

ABBREVIATIONS: **AAE:** ascorbic acid equivalents; **Aq:**aqueous; **Bec:** cap; **Bes:** stipe; **DMEM:** Dulbecco's modified Eagle's medium; **DMSO:** dimethyl sulfoxide; **DPPH•:**2,2 – diphenyl – 1-picrylhydrazyl; **EtOH:**ethanol; **FC:**FolinCiocalteu; **FCS:**fetal calf serum; **FRAP:** Ferric reducing antioxidant power; **GAE:** gallic acid equivalents; **IC_{50}:**half-maximal inhibitory concentration values; **MCF-7:** breast carcinoma cell line; **MDA:** malondialdehyde; **MTT:**3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide; **NADH:** nicotinamide adenine dinucleotide; **NEDA:** naphthylethylenediamine dihydrochloride; NO**•:** nitric oxideradical; **OH•:**hydroxyl radical; **PMS:** phenazine methosulfate; **ROS:**reactive oxygen species; **SA:** sulfanilamide; **SNP:** sodium nitroprusside dihidrate; **SO•:** superoxide anion radical; **TBA:** 2-thiobarbituric acid; **TF:** total flavonoid; **TP:** total phenolic

I. INTRODUCTION

Since ancient times, mushrooms have been mainly used as special food, due to their high nutritional value, undoubtedly fine flavors, as well as their pronounced health beneficial properties[1,2].

Basidiomycete family Boletaceae aremushrooms that are mainly known by fleshy context and a tubulose, rarely lamellate or loculate hymenophore. This family has extraordinary diversity which includes about 50 genera and 800 species[3]. According to recent phylogenetic analyses, seven major clades at the subfamily level have been revealed, namely Austroboletoideae, Boletoideae, Chalciporoideae, Leccinoideae, Xerocomoideae, Zangioideae, and the Pulveroboletus group[4]. Some of these boletes have great economic, dietary and health value such as *Boletus edulis* sensu lato (Porcini) which is a gourmet mushroom highly prized in many parts of the world, including the Balkans region. This species is widely distributed in the across Europe, Asia and North America, but it is also one of the most popular mushroom species in Serbian cuisine. Among the species within genus *Boletus*, there are many species which are consumed in ourregion such as *B. aereus, B. aestivalis, B. erythropus, B. pinicola*, *B. queletii* etc[5].Within the local edible species in Serbia, *B. edulis*, stands out due to high consumption and its economic value. The mushroom is low in fat and digestible carbohydrates and high in protein, vitamins, minerals and dietary fiber. Although it is sold commercially, it is very difficult to cultivate due to its mychorizal needs. It is available in wild, and it is most often dried, packaged and distributed worldwide. After drying, it keeps flavor which makes it suitable as milled powder to be used in cooking[6,7,8].

This species produces a variety of nutraceuticals, with a diverse spectrum of biological activity such as anti-oxidant, anticancer, anti-microbial[9,10,11]. The bioactivity of Porcini was first confirmed by Lucas in 1957, when he isolated a substance from *B. edulis* which showed a significant inhibitory effect against tumor cells of sarcoma (S-180)[12,13].

Free radicals are reactive oxygen species (ROS) in cells, which are constantly produced in the human body [14]. There isa lot of evidence that supports the implication of oxidative stress inducedby reactive oxygen species (DPPH•NO•, SO•, OH•) in the pathogenesis of several chronic and degenerative diseases such as: Alzheimer's, Parkinson's, atherosclerosis, diabetes mellitus, chronic inflammation and cancer. Therefore, the enhancement of the antioxidant systems for the prevention of cellular oxidative stressvia the consumption of antioxidant rich foods is of great interest today[14,15,16,17,18,19].

Since the bioactivity of fungal species is mostly influenced by geographical origin or specificity of the single fungal strain[20] the aim of this study was to evaluate antioxidant and antiproliferative properties of aqueous and ethanolic crude extracts of caps and stipes of autochthonous *B. edulis* species with respect to their total phenolic and flavonoid contents.

Moreover, based on the literature data concerning antioxidant properties obtained for different parts of fruiting bodies **(**cap and stipe) the aim of this study was also to make a comparison between bioactive properties of cap and stipe of autochtonous mushroom species *B. edulis*.

To the best of our knowledge, this paper presents the first report concerning antioxidant and cytotoxic potential of the tested species from Western Serbian region of Prijepolje. Furthermore, for the first time ethanolic and aqueous extracts of cap and stipe were analyzed against MCF-7 breast carcinoma cell line.

II. MATERIALS AND METHODS

A. Materials and Chemical

B. edulis was collected from Prijepolje region (Western Serbia) during autumn 2011. After its identification, a voucher specimen was deposited at the Herbarium BUNS, Novi Sad, Serbia. The fungal samples were separated to the cap (Bec) and stipe (Bes) parts and frozen at -20°C, prior to freeze-drying procedure (Bio alpha, Martin Christ GmbH, Germany). Freeze-dried samples were ground to a fine powder, wrapped in plastic bags and stored in dark place at room temperature, until further use. Folin-Ciocalteu (FC) reagent, anhydrous sodium carbonate, gallic acid, aluminium (III) chloride hexahydrate, sodium acetate trihydrate, quercetin hydrate, 2,2 – diphenyl – 1-picrylhydrazyl (DPPH•), anhydrous iron(III) chloride (FeCl3), disodium hydrogen phosphate, phenazine methosulfate (PMS), and β-nicotinamide adenine dinucleotide (NADH), malondialdehyde (MDA), 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT), dimethyl sulfoxide (DMSO), were purchased from Sigma-Aldrich (Steinheim, Germany). Dulbecco's Modified Eagle's Medium with 4.5% of glucose (DMEM) and fetal calf serum (FCS) were purchased from PAA Laboratories (Pasching, Austria). Ascorbic acid, hydrochloric acid (HCl), potassium dihydrogen phosphate, sodium nitroprusside dihidrate (SNP), naphthylethylenediamine dihydrochloride (NEDA),

and sulfanilamide (SA) were purchased from Lach-ner (Neratovice, Czech Republic). Deionized water was produced using a Millipore water purification system.

B. Preparation of ethanolic and aqueous extracts from *B. edulis*

The processed caps and stipes of *B. edulis* (10 g) were extracted with distilled water (Aq) and ethanol (EtOH) for 24h, on a shaker (Thermo Fisher Scientific, USA; 120 rpm) at room temperature (25°C). The extracts were filtered through Whatman No. 4, while the solvents (EtOH) were removed by rotary evaporator unit at 40°C (Bűchi, Switzerland); the aqueous extract (BecAq, BesAq) was freeze-dried prior to analysis. The obtained extracts (ethanolic -BecEtOH, BesEtOH and aqueous BecAq, BesAq) were stored at +4°C and -20°C, respectively. Relevant dried residues were re-dissolved in 5% DMSO.

C. Total phenolic content (TP)

Total phenolic content (TP) of all analyzed extracts was determined according to method by Singleton et al. [22], adapted for a 96-well plate reader (Multiskan Ascent, Thermo Electron Corporation, USA). Folin-Ciocalteu reagent (125 µl, 0.1 M) was added to 25 µl of diluted extracts. After 10 min, 100 µl of 7.5% w/v sodium carbonate was added and reaction mixture was incubated for 2 h. Absorbance was read at 690 nm. TP was expressed as mg gallic acid equivalents (GAE)/g of dry weight (d.w.). The experiments were performed in triplicate.

D. Total flavonoid content (TF)

166 Total flavonoid (TF) content of all extracts was measured spectrophotometrically,
167 in a 96-well plate reader, using a modified method by Chang et al[23]. The relevant
168 sample (30 µl) was mixed with 90 µl of methanol, 6 µl of 0.75 M aluminium-
169 trichloride, 6 µl of 1 M sodium acetate and 170 µl of distilled water. Absorbance
170 was measured at 414 nm, after incubation of 30 min. The experiments were
171 repeated three times, while results were expressed as mg quercetin equivalents
172 (QE)/g of dry weight (d.w.).

173 **E. Determination of DPPH•, NO•, SO• and OH• scavenging activity**

174 DPPH• scavenging activity was evaluated according to Espin et al.[24]. The
175 reaction mixture consisted of 10 µl of sample, 60 µl of DPPH• solution and 180 µl
176 of methanol. After incubation of 60 min (dark place, at room temperature),
177 absorbance was measured at 540 nm. Each sample was tested at different
178 concentrations in the range (15 – 600 µg/ml), while results were expressed as IC_{50}
179 value.

180 Nitric oxide scavenging capacity was determined according to the
181 procedure of Green et al.[25]. The reaction mixtures in test tubes consisted of 30 µl
182 extract, 500 µl SNP, and 500 µl of phosphate buffer, pH=7.4. Control contained
183 equivalent volume of ethanol, while reagents were replaced with phosphate buffer
184 in the correction. Test tubes were incubated at room temperature for 90 min, under
185 light exposure. After incubation, 1 ml of Griess reagent (0.2% solution of NEDA
186 and 2% solution of SA in 4% phosphoric acid in the ratio 1:1 (v:v) was added
187 equally to samples, corrections and control. Aliquots of 250 µl were transferred to
188 the plate, and their absorbance was measured using plate reader at 540 nm.
189 Samples were tested at different concentrations (0.9 – 1477 µg/ml) to obtain IC_{25}.

Superoxide anion radical scavenging capacity of extracts was determined by measuring their ability to neutralize superoxide anion radicals generated during aerobic reduction of nitro blue tetrazolium by NADH mediated by PMS[26]. Total amount of 100 µl of 677 µM NADH, 100 µl of 60 µM PMS, 200 µl of 144 µM NBT and 1,1 ml of phosphate buffer (pH = 8.3) were mixed with 10 µl of extract in the test tube. Control contained ethanol instead of extract, and correction contained 10 µl of extract and 1,5 ml of phosphate buffer. After 5 min, aliquots of 250 µl were transferred to the well plate and their absorbance was measured at 540 nm using plate reader. Five different concentrations of each sample (1.65 – 99.3 µg/ml) were tested to obtainIC_{50}.

The content of OH radicals was determined from the degradation reaction of 2-deoxy-D-ribose into fragments[27], while the malondialdehyde MDA reaction with 2-thiobarbituric acid TBA reagent was determined spectrophotometrically at 532 nm. Each reaction was performed in triplicate using 10 µL of fungal extract (0.98 – 28.79 µg/mL).

All results were calculated to obtain IC_{50}, and all experiments were performed in triplicate.

F. Ferric reducing antioxidant power (FRAP)

FRAP assay was performed according to a modified procedure of Benzie and Strain[28]. The FRAP reagent consisted of 300 mM acetate buffer (pH=3.6), 10 mM TPTZ in 40 mM HCl and 20 mM $FeCl_3$, in the ratio 10:1:1 (v:v:v). The sample (10 µl), 225 µl of FRAP reagent and 22.5 µl of distilled water were added in a 96-well plate. Absorbance was measured after 6 min at 620 nm. The results were

213 expressed as mg ascorbic acid equivalents (AAE)/g of dry weight (d.w.). Each
214 analysis was performed three times.

215 **G. Antiproliferative activity**

216 **1. Cells**

217 Estrogen dependent MCF-7 cells were grown in DMEM supplemented with 10%
218 FCS. The cells were seeded in a 96-well microplate (5000 cells per well). After
219 incubation for24 h, the growth medium was replaced with 100 μl of medium
220 containing examined samples (extracts) at four different concentrations (33.3
221 μg/ml, 100.0 μg/ml, 300.0 μg/ml, and 900.0 μg/ml). Untreated cells served as
222 control, while pure DMSO was used as a positive control. The effects of the
223 extracts on the growth of MCF-7 cells were evaluated by standard colorimetric
224 assay – MTT.

225 **2. MTT Assay**

226 After 24 h and 72 h respectively, the cell viability was determined by the
227 proliferation MTT assay[29]. This assay is based on the color reaction of
228 mitochondrial dehydrogenase in living cells with MTT reagent. After incubation,
229 MTT reagent was added to each well (50 μg/100 μl/well at 37 °C, in 5% CO_2 for 3
230 h). The crystals of produced formazan were dissolved in 100 μl of acidified
231 isopropanol (0.04 M HCl in isopropanol). Absorbance was measured at 540 nm
232 and 690 nm on a 96 well plate reader (Multiskan Ascent, Thermo Electron
233 Corporation, USA).

234 Calculation of cytotoxicity is expressed as: a percentage of corresponding control
235 value (non-treated cells) obtained in a minimum of three independent experiments.
236 The half-maximal inhibitory concentration values (IC_{50}), defined as the

concentration that inhibits 50% of cell growth, were calculated from concentration-response curves. IC_{50} values were determined in accordance with dose dependent effects and dose-response curves. IC_{50} values were expressed as the mean value of a minimum of three repeated experiments performed for each extract.

H. Statistical analysis

Statistical analysis was performed using Statistica software system (StatSoft, Inc. version 12.0, 2013)[30]. Significant differences between two groups were determined by Duncan's multiple range tests. Finally, Pearson correlation was calculated for TP and TF, DPPH•, NO•, SO•, OH•, FRAP and IC_{50} values for antiproliferative activity.

III. RESULTS AND DISCUSSION

A. Antioxidant activity and total phenolic and flavonoid contents

Scavenging effects of examined extracts from *B. edulis* on DPPH• increased with increased extract concentrations. For scavenging ability on DPPH radicals, extracts were effective in the following order of activities: BecAq> BesEtOH >BesAq>BecEtOH. Among the examined extracts, IC_{50} value of BecAq stood out (50.97 μg/ml) in DPPH• scavenging assay (Table 1). In general, analysis showed that aqueous extracts of cap and stipe had higher activities in DPPH assay than experimental data obtained for *B. edulis* extracts of cap and stipe originated from Portugal[21] and lower activities than ethanol extracts of the entire mushroom originated from Istra region of Croatia[31]. Extracts of *B. edulis* showed good scavenging activity on nitric oxide, where BesAq extract showed the highest

activity expressed as IC_{25} at 10.74 µg/ml while all extracts were effective in the following order: BesAq > BecAq > BesEtOH > BecEtOH. The radical scavenging activity on SO•, exhibited relatively high level at lower concentration (IC_{50} BesEtOH was at 8.55 µg/ml) while the extracts were effective in the following order: BesEtOH > BesAq >BecAq > BecEtOH.

All analyzed extracts were able to scavenge hydroxyl radicals (BecAq > BecEtOH > BesAq > BesEtOH) (Table 1), although BecAq extract showed the highest activity (IC_{50}=2.61 µg/ml).These results showed more potent values than those reported by previous researches on various extracts from *B. edulis*[32]. Moreover, all analyzed extracts showed very good scavenging activity towards SO• and OH radical, although IC_{50} values did not differ significantly between them.

Furthermore the aqueous extract obtained from the mushroom cap showed also the highest ferric reducing antioxidant power (FRAP) (22.14 mg/g): BecAq > BesEtOH > BecEtOH > BesAq, although IC_{50} did not differ significantly.

Total phenolic (TP) content was in the range of 29.18–36.36 mg/g although it did not differ significantly (Table 2).These results are in accordance with previous studies on *B. edulis* samples from India, Taiwan, Turkey and Portugal[16,33,34,35]. The examined EtOH extracts in this study had slightly lower TP than ethanolic extracts of *B. edulis* samples from Istra region of Croatia[31]. TF contents were found to be in the range of 0.65 – 4.50mg/g and BesAq extract showed significantly higher concentration. The highest antioxidant values, including antiradical (the lowest IC_{50} values) were found for the BecAq, which is in agreement with its highest TP and TF content.

B. Antiproliferative activity

285 The antiproliferative activity of extracts from *B. edulis* was screened by the MTT
286 assay on human breast carcinoma cells (MCF-7). It has been previously reported[36]
287 that compounds exhibiting cytotoxic effects in cell lines can demonstrate distinct
288 kinetic profiles that fit into three categories: acute (< 1h to full toxicity), subacute
289 (1 - 40h), and long term (>40h). Guided by these findings, exposure times of 24 h
290 and 72h have been selected to allow the expression of full cytotoxic potential of
291 investigated fungal extracts.
292 Under the experimental conditions all extracts showed cytotoxic activity (Table 3).
293 At the highest tested concentration (900 μg/ml), cytotoxicity of EtOH extracts was
294 expressed in the range of 45% - 94%, while for Aq extracts it was in the narrow
295 range of activities between 32% - 84%. Ethanolic extracts from stipe (BesEtOH)
296 elicited very prominent antiproliferative effect (IC_{50}=59.14 μg/ml). This extract
297 showed significantly higher activity (lower IC_{50}) than aqueous extract especially
298 for the chronic treatment at 72 h (Table 3). The aqueous extracts from cap
299 (BecAq) induced less pronounced cytotoxicity (with IC_{50}>900 μg/ml). However,
300 when comparing our results with the findings from the similar studies,
301 antiproliferative potency of *B. edulis* on the human breast cancer cell line (MCF-7)
302 is evident.
303 The mushroom biopolymers (polysaccharides and glycoproteins) isolated from *B.*
304 *edulis* hot water extract showed very prominent antiproliferative effect in colon
305 cancer cells (CCD 841 CoTr) and no toxic effect against normal colon epithelial
306 cells[18]. The novel lectin from *B. edulis* was found to effectively inhibit the
307 proliferation MCF-7 (breast adenocarcinoma), HepG-2 (hepatocellular carcinoma),
308 CaCo-2 (colorectal adenocarcinoma), CFPAC-1(pancreatic duct adenocarcinoma),

309 HeLa, SK-MEL-28, U-87 MG cell lines[19].On the other hand three non-isoprenoid
310 botryane sesquiterpenoids named boledulins A–C (1–3) isolated from the cultures
311 of *B. edulis* exhibited inhibitory activity against five human cancer cell lines: HL-
312 60, SMMC-7721, A-549, MCF-7 and SW480[37].
313 The observed difference in antioxidant and antiproliferativeactivity of *B. edulis*
314 extracts might depend on their chemical composition. Mushrooms contain a
315 variety of complex compounds derived from secondary metabolism such as
316 phenolic compounds, polyketides, triterpenoids and steroids which are specific to
317 each mushroom and have specific effects on humans[38,13,9,40] Phenolic compounds
318 in mushrooms and in other sources display a remarkable spectrum of biological
319 activities including antioxidant, antitumor and antimicrobial
320 properties[41,42,43,44,45,46,47].
321 Protocatechuic acid, *p*-hydroxybenzoic acid, *p*-coumaric acid, cinnamic acid, and
322 quinic acid were previously found in some *B. edulis* extracts from Portugal and
323 Serbia[35,47]and variagatic acid in extracts from Istra region of Croatia[31].These
324 phenolic acids possess several bioactivities, such as antioxidant[41], cytotoxic[48],
325 antiradical[42] and antitumor[49]. Heleno et al.[45] referred the cytotoxic and antitumor
326 activities of *p*-coumaric acid against breast MCF-7, NCI-H460 and HCT15
327 carcinoma cell lines and also referred to the antioxidant activities. In addition,
328 anticancer studies *in vitro* and *in vivo* pointed to flavonoids as major compounds,
329 against cancer which prevent carcinogen metabolic activation, antiproliferation,
330 cell cycle arrest, induction of apoptosis, promotion of differentiation, antioxidative
331 activity and modulation of multidrug resistance[50].

Correlation between TP and TF content and tested antiproliferative activity (IC_{50} value) among all applied tests showed much higher values (r=0.806, $p<0.05$) for TF content, especially for treatment at 24 h (Fig. 1) than for TP content (r=0.49, $p<0.05$), and for DPPH assay (r=-0.80, $p<0.05$) (Fig. 2).This suggests an important role of flavonoid compounds in demonstrated antiproliferative activities and radical scavenging activities on DPPH.

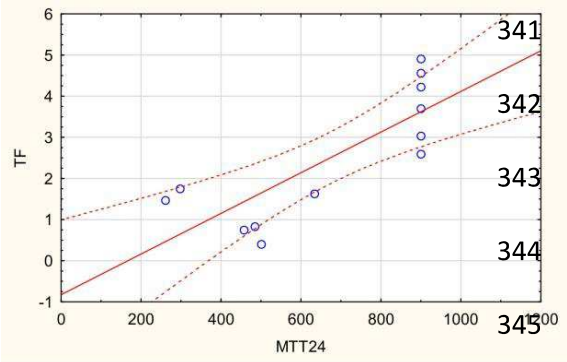

FIG. 1: Correlation between TP and TF content and tested antiproliferative activity (IC_{50} value).

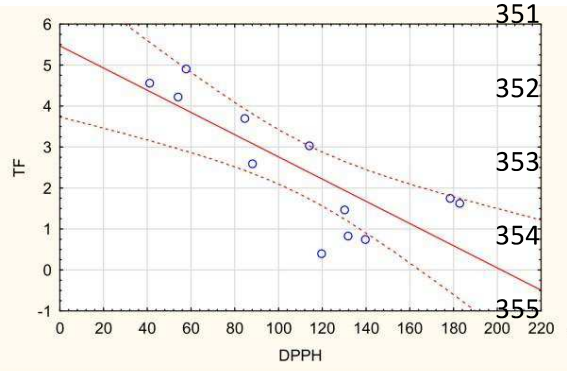

FIG.2: Correlation between TF content and scavenging ability on DPPH radicals (IC_{50} value).

IV. CONCLUSIONS

According to the results of this study, it is clearly indicated that the tested mushroom extracts of *B. edulis,* especially aqueous extract from cap have strong antioxidant activity *in vitro*, including antiradical and FRAP activity. Cytotoxicity assay proved that crude ethanolic extract from stipe possesses the highest antiproliferative activity. According to the results obtained the examined fungal species herein, may be considered as a promising source of natural antioxidants with potential significance for regular human diet. Considering autochthonous origin of wild-growing sporocarps of *B. edulis*, demonstrated activities are even more important for the region and for the further exploitation. The future research work should be primarily directed towards elucidation of chemical profiles of analyzed extracts derived from cap and stipe and their possible mechanism(s) of action.

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REFERENCES

1. Wasser SP. Current findings, future trends, and unsolved problems in studies of medicinal mushrooms. Appl Microbiol Biotechnol. 2011; 89(5):1323-1332.

2. Wani BA, Bodha RH, Wani AH. Nutritional and medicinal importance of mushrooms. J Med Plants Res. 2010; 4(24):2598-604.

3. Kirk PM, Cannon PF, Minter DW, Stalpers JA. Dictionary of the Fungi. 10 ed. Wallingford, UK. 2008

4. Wu G, Feng B, Xu J, Zhu XT, Li YC, Zeng NK,Yang ZL. Molecular phylogenetic analyses redefine seven major clades and reveal 22 new generic clades in the fungal family Boletaceae. Fungal Divers. 2014;69(1): 93-115.

5. Uzelac B. Mushrooms of Serbia and the Western Balkans. BGV Logik, Serbia: 2009.

6. Hall IR, Lyon AJ, Wang Y, Sinclair, L. Ectomycorrhizal fungi with edible fruiting bodies 2. Boletus edulis. Econ bot. 1998;52(1):44-56.

7. Polese JM, Lamaison JL. The Great Encyclopedia of Mushrooms.1999

8. Dimitrijevic M, Jovanovic VS, Cvetkovic J, Mihajilov-Krstev T, Stojanovic Mitic V. Screening of antioxidant, antimicrobial and antiradical activities of twelve selected Serbian wild mushrooms. Anal Methods. 2015; 7(10):4181-4191.

9. Ivanova TS, Krupodorova TA, Barshteyn VY, Artamonova AB, Shlyakhovenko VA. Anticancer substances of mushroom origin. Exponcol. 2014; 36 (2):58-66.

10. Patel S, Goyal A. Recent developments in mushrooms as anti-cancer therapeutics: a review. 3 Biotech 2012; 2:1-15.

11. Khatun S, Islam A, Cakilcioglu U,Chatterje NC. Research on mushroom as a potential source of nutraceuticals: A review on Indian perspective. American Journal of Experimental Agriculture. 2012;
12. Lucas EH, Ringler RL, Byerrum RU, StevensJA, Clarke DA, Stock CC. Tumor inhibitors in Boletus edulis and other Holobasidiomycetes. Reprint from Antibiot. & Chemother.1957;7(1).
13. Zhang M, Cui SW, Cheung PCK, Wang Q. Antitumor polysaccharides from mushrooms: a review on their isolation process, structural characteristics and antitumor activity. Trends Food SciTech.2007; 18(1):4-19.
14. Ferreira IC, Barros L, Abreu R. Antioxidants in wild mushrooms. Curr Med Chem.2009;16(12):1543-1560
15. Pham-Huy LA, He H, Pham-Huy C. Free radicals, antioxidants in disease and health. Int J Biomed Sci. 2008;4(2):89-96
16. Halliwell B. Antioxidants in human health and disease. Ann Rev Nutr. 1996;16(1):33-50.
17. Valko M, Leibfritz D, Moncol J, Cronin MT, Mazur M, Telser J.Free radicals and antioxidants in normal physiological functions and human disease. Int J Biochem Cell Biol. 2007;39(1):44-84.
18. Lemieszek MK, Cardoso C, Nunes FHFM, Marques G, Pożarowski P, Rzeski W. Boletus edulis biologically active biopolymers induce cell cycle arrest in human colon adenocarcinoma cells. Food funct. 2013;4(4):575-585
19. Bovi M, Cenci L, Perduca M, Capaldi S, Carrizo ME, Civiero L, Monaco HL. BEL β-trefoil: A novel lectin with antineoplastic properties in king bolete (Boletus edulis) mushrooms. Glycobiology. 2013;23(5):578-592.
20. Puttaraju NG, Venkateshaiah SU, Dharmesh SM, Urs SMN, Somasundaram R, Antioxidant activity of indigenous edible mushrooms. J Agric Food Chem. 2006;54(26):9764-9772

21. Ribeiro B, Lopes R, Andrade PB, Seabra RM, Gonçalves RF, Baptista P, Quelhas I. Comparative study of phytochemicals and antioxidant potential of wild edible mushroom caps and stipes. Food Chem. 2008;110(1):47-56

22. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of folin-ciocalteu reagent. Method Enzymol. 1999;299:152-178.

23. Chang CC, Yang MH, Wen HM, Chern JC. Estimation of total flavonoid content in propolis by two complementary colorimetric methods. J Food Drug Anal. 2002; 10(3):178-182.

24. Espín JC, Soler-Rivas C, Wichers HJ. Characterization of the total free radical scavenger capacity of vegetable oils and oil fractions using 2, 2-diphenyl-1-picrylhydrazyl radical. J Agric Food Chem. 2000;48(3):648-656.

25. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR. Analysis of nitrate, nitrite, and [15N] nitrate in biological fluids. Analy Biochem. 1982;126(1):131-138.

26. Nishikimi M, Rao NA, Yagi K. The occurrence of superoxide anion in the reaction of reduced phenazine methosulfate and molecular oxygen. Biochem Biophys Res. 1972;46(2):849-854.

27. Cheeseman KH, Beavis A, Esterbauer H. Hydroxyl-radical-induced iron-catalysed degradation of 2-deoxyribose. Quantitative determination of malondialdehyde. Biochem. J. 1988;252:649-653.

28. Benzie IF, Strain JJ. Ferric reducing/antioxidant power assay: Direct measure of total antioxidant activity of biological fluids and modified version for simultaneous measurement of total antioxidant power and ascorbic acid concentration. Method Enzymol. 1998;299:15-27.

455 29. Mosmann T. Rapid colorimetric assay for cellular growth and survival:
456 application to proliferation and cytotoxicity assays. J Immunol Methods.
457 1983;65(1-2):55-63.
458 30. StatSoft, Inc. version 12.0, 2013
459 31. Vidović SS, Mujić IO, Zeković ZP, Lepojević ŽD, Tumbas VT, Mujić AI.
460 Antioxidant properties of selected Boletus mushrooms. Food Biophys.
461 2010;5(1):49-58.
462 32. Vamanu E, Nita S. Antioxidant capacity and the correlation with major phenolic
463 compounds, anthocyanin, and tocopherol content in various extracts from the wild
464 edible Boletus edulis mushroom. Bio Med Res Int. 2012;2013.
465 33. Tsai SY, Tsai HL, Mau JL. Antioxidant properties of Agaricus blazei, Agrocybe
466 cylindracea, and Boletus edulis. LWT-Food Sci Technol. 2007;40(8):1392-1402.
467 34. Sarikurkcu C, Tepe B, Yamac M. Evaluation of the antioxidant activity of four
468 edible mushrooms from the Central Anatolia, Eskisehir–Turkey: Lactarius
469 deterrimus, Suillus collitinus, Boletus edulis, Xerocomus chrysenteron.
470 Bioresource Technol. 2008;99(14):6651-6655.
471 35. Heleno SA, Barros L, Sousa MJ, Martins A, Santos-Buelga C, Ferreira IC.
472 Targeted metabolites analysis in wild Boletus species. LWT-Food Sci Tech.
473 2011;44(6):1343-1348
474 36. Xia M, Huang R, Witt KL, Southall N, Fostel J, Cho M, Tice RR. Compound
475 cytotoxicity profiling using quantitative high-throughput screening. Environ
476 Health Persp. 2008;116(3):284
477 37. Feng T, Li ZH, Dong ZJ, Su J, Li Y, Liu JK. Non-isoprenoid botryane
478 sesquiterpenoids from basidiomycete Boletus edulis and their cytotoxic activity.
479 Nat prod bioprospect. 2011;1(1):29-32.

480 38. De Silva DD, Rapior S, Sudarman E, Stadler M, Xu J, Alias SA, Hyde KD.
481 Bioactive metabolites from macrofungi: ethnopharmacology, biological activities
482 and chemistry. Fungal Divers. 2013;62(1):1-40.
483 39. Wasser SP. Medicinal mushroom science: history, current status, future trends,
484 and unsolved problems. Int J Med Mushrooms. 2010;12(1)
485 40. Ferreira CFR, Vaz I, Vaz AJ, Vasconcelos MH, Martins A. Compounds from wild
486 mushrooms with antitumor potential. Anti-Cancer Agents Med Chem.
487 2010;10(5):424-436.
488 41. Karaman M, Stahl M, Vulić J, Vesić M, Čanadanović-Brunet J. Wild-growing
489 lignicolous mushroom species as sources of novel agents with antioxidative and
490 antibacterial potentials. Int J Food Sci Nutr. 2014;65(3):311-319.
491 42. Ferreira IC, Barros L, Abreu R Antioxidants in wild mushrooms. Curr Med Chem.
492 2009; 16(12):1543-1560
493 43. Rice-Evans CA, Miller NJ, Paganga G. Structure-antioxidant activity relationships
494 of flavonoids and phenolic acids. Free Radic Biol Medicine. 1996; 20(7): 933-956
495 44. Carocho M, Ferreira CFR. The role of phenolic compounds in the fight against
496 cancer–a review. Anti-Cancer Agents Med Chem. 2013;13(8):1236-1258.
497 45. Heleno SA, Martins A, Queiroz MJR, Ferreira IC. Bioactivity of phenolic acids:
498 Metabolites versus parent compounds: A review. Food chem. 2015;173:501-513.
499 46. Alves MJ, Ferreira IC, Dias J, Teixeira V, Martins A, Pintado M. A review on
500 antimicrobial activity of mushroom (Basidiomycetes) extracts and isolated
501 compounds. Planta Med. 2012;78:1707-1718
502 47. Stojković DS, Reis FS, Ćirić A, Barros L, Glamočlija J, Ferreira IC, Soković M.
503 Boletus aereus growing wild in Serbia: chemical profile, in vitro biological
504 activities, inactivation and growth control of food-poisoning bacteria in meat. J
505 Food Sci Tech. 2015;52(11):7385-7392

48. Yip ECH, Chan ASL, Pang H, Tam YK,Wong YH. Protocatechuic acid induces cell death in HepG2 hepatocellular carcinoma cells through a c-Jun N-terminal kinase-dependent mechanism. Cell Biol Toxicol. 2006;22(4):293-302

49. Vaz JA, Almeida GM, Ferreira IC, Martins A, Vasconcelos MH. Clitocybe alexandri extract induces cell cycle arrest and apoptosis in a lung cancer cell line: identification of phenolic acids with cytotoxic potential. Food Chem. 2012; 132(1):482-486.

50. Ren W, Qiao Z, Wang H, Zhu L, Zhang L. Flavonoids: promising anticancer agents. Med Res Rev. 2003;23(4):519-534.

TABLE 1:Bioactivity of different extracts of *B.edulis*

	BecEtOH	BecAq	BesEtOH	BesAq
***Antiradical assays IC_{50} (µg/ml)**				
$DPPH^{\bullet}$	163.83^{c}	50.97^{a}	95.60^{b}	130.42^{c}
$^{**}NO^{\bullet}$	1034.88^{c}	$433.97^{a,b}$	1212.16^{c}	10.74^{a}
$SO^{\bullet}$	18.38^{a}	12.36^{a}	9.84^{a}	10.55^{a}
$OH^{\bullet}$	3.75^{a}	2.05^{a}	30.0^{a}	5.28^{a}
***Antioxidant assay**				
$FRAP^{***}$	12.59^{a}	15.13^{a}	22.14^{a}	8.43^{a}

**expressed as concentration of extracts that caused 50% activity - IC_{50} (µg/ml)*

***expressed as concentration of extracts that caused 25% activity – IC_{25} (µg/ml)*

****Ferric reducing antioxidant power (FRAP) is expressed as mg ascorbic acid equivalents/g extract dry weight (mg AAE/g d.w.)*

^{a,b,c} Significant differences between groups (columns) were determined by Duncan's test ($p<0.05$)

TABLE 2: Chemical composition of different extracts of *B.edulis*

Total content	BecEtOH	BecAq	BesEtOH	BesAq
TP*	23.94[a]	34.73[a]	36.36[a]	29.18[a]
TF*	1.61[c]	4.50[d]	3.10[a]	0.65[b]

**Total phenolic (TP) content is expressed as mg gallic acid equivalents/g extract dry weight (mg GAE/g d.w.), while total flavonoid (TF) content is expressed as mg quercetin equivalents/g extract dry weight (mg QE/g d.w.)*

[a,b,c] Significant differences between groups (columns) were determined by Duncan's test ($p<0.05$)

558 **TABLE 3: Antiproliferative activity of *B. edulis* extracts on MCF-7 cell line**

	MTT assay IC_{50} (µg/ml)	
Extracts	**24h**	**72h**
BecEtOH	$397.80^{a} \pm 68.42$	$163.82^{b} \pm 4.20$
BecAq	$900^{b} \pm 0.00$	$900^{d} \pm 0.00$
BesEtOH	$900^{b} \pm 0.00$	$59.14^{a} \pm 6.66$
BesAq	$481.48^{a} \pm 7.25$	$435.61^{c} \pm 15.14$

559 $^{a,b,\cdots}$ *Values with different letters in superscript within a row are statistically*
560 *different ($p<0.05$), Duncan's test.*

561 **FIG. 1: Correlation between TP and TF content and tested antiproliferative**
562 **activity (IC_{50} value).**

563 **FIG. 2: Correlation between TF content and scavenging ability on DPPH**
564 **radicals (IC_{50} value).**

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