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NUTRITIONAL AND ANTIOXIDANT PROFILE OF THE MEDICINAL MUSHROOMS *PHELLINUS TORULOSUS* AND *P. IGNIARIUS*: INFLUENCE OF DIFFERENT EXTRACTANTS ON BIOACTIVITY

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The increased demand for natural, safe and dietary antioxidant sources stimulated the exploration of *in-vitro* reducing and scavenging potentials of extracts from wild medicinal and non-toxic mushrooms with intention of acknowledging their therapeutic values. Based on the findings about enhanced potency of extracts *vs.* powder, as well as highlighting the antioxidant potential of underexploited wild species from Macedonian territory, our investigations were aimed at screening the reducing and scavenging profiles of hot water (HWEs), cold water (CWEs) and methanol extracts (MEs) from *Phellinus torulosus* and *P. igniarius* to promote their usage as nutraceuticals and for medicinal use. MEs from both species exhibited superior scavenging activity over radicals: 2,2-diphenyl-1-picrylhydrazyl (DPPH), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and nitric oxide (NO); enhanced reducing properties and higher total antioxidant capacity, owed to their significantly higher phenol and flavonoid content. In contrast, CWEs established superior ability for inhibiting the lipid peroxides level and glycosylated protein adducts but exposed lower antioxidant properties and reducing abilities. HWE from *P. torulosus* was a more efficient radical scavenger than *P. igniarius*, with higher reducing and *in-vitro* antiglycation capacities, thereby proving its suitability for the treatment against disorders induced by oxidative stress.

Keywords: medicinal fungi; extraction; bioactive content, antiradical activity

НУТРИТИВЕН И АНТИОКСИДАЦИСКИ ПРОФИЛ НА МЕДИЦИНСКИТЕ ПЕЧУРКИ *PHELLINUS TORULOSUS* И *P. IGNIARIUS*: ВЛИЈАНИЕ НА РАЗЛИЧНИ ЕКСТРАКТАНТИ ВРЗ БИОАКТИВНОСТА

Зголемената потреба од природни, безбедни и диетни извори на антиоксиданси ја поттикна целта за евалуација на редуктивните и неутрализирачки потенцијали на екстракти од диви и нетоксични медицински печурки, мерени во *in vitro* услови, со цел утврдување на нивните терапевтски вредности. Земајќи ги предвид податоците од претходните истражувања кои укажуваат на поголема антиоксидативна активност на екстрактите, наспроти прашестите суплементи, како и потребата од истакнување на нутритивната вредност и потенцијалот на ретки и неистражени медицински габи од територијата на РС Македонија, нашите истражувања беа фокусирани на утврдување на типот на биоактивните компоненти, нивниот сооднос и неутрализирачките својства на екстракти од габите *Phellinus torulosus* и *P. igniarius* во различни растворувачи или различен начин на подготовка: во вриечка дејонизирана вода (HWE); ладна дејонизирана вода (CWE), како и во метанолни екстракти (ME). Добиените резултати покажаа дека ME од двата вида поседуваат сигнификантно поизразена редуктивна и вкупна антиоксидативна моќ, повисока неутрализирачка активност врз радикалот 2,2–дифенил-1-пикрилхидразил (DPPH); изразена неутрализација врз 2,2–азинобис(3-етилбензотиалин-6-сулфонска киселина) (ABTS), но и моќ за неутрализација на азотниот моноксид (NO), што беше во корелација со утврдената содржина на феноли и флавоноиди во користените екстракти. Наспроти

метанолните, водните екстракти (CWE) поседуваа висок потенцијал за инхибиција на липидната пероксидација и инхибиција врз процесот на гликација на протеинските адукти, но тие покажаа и пониски примарни антиоксидативни карактеристики. HWE од габата *P. torulosus* покажа подобра способност за неутрализација врз радикалот ABTS, појака редуктивна активност и поизразен антигликациски ефект во однос на *P. igniarius*, истакнувајќи ги неговите терапевтски можности против заболувања предизвикани од оксидативно оштетување.

Клучни зборови: медицински габи; екстракција; биоактивен потенцијал; неутрализирачка моќ

1. INTRODUCTION

Mushrooms are a nutritionally resourceful food. They are a source of valuable high protein content, significant fiber and indispensable amino acids, which make them highly attractive cosmeceuticals and pharmaceuticals. However, evidence regarding the presence of bioactive metabolites with immense therapeutic properties, in recent years, have promoted the use of edible and wild mushrooms as safe natural antioxidants that play an important role in prevention of oxidative stress, cellular damage and immunodeficiency.[1,2] Despite their utilization as traditional remedies, several species with strong chelating and scavenging characteristics were also included as safe and functional prophylactics in modern medicine suppressing diabetes, hypertension or cancer.[3,4] Moreover, non-toxic mushrooms could be an essential part of healthy diets due to their rich nutritive value, distinctive flavor and significant dietary fiber content in their fruiting bodies (35–70 % dry weight).[5]

Phellinus mushrooms are among the medicinal basidiomycetes with rich bioactive content and potent antioxidant properties used against a variety of disorders as documented in oriental medicine.[6] Studies confirmed their significant 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity, inhibition of lipid peroxidation and reducing power, as well as antibacterial and antidiabetic actions as determined for *P. baumii* Pilát,[7] anti-inflammatory activities confirmed for *P. rimosus* (Berk.) Pilát [8], and anticancer properties verified only for *P. linteus* (Berk. & M.A. Curtis) Teng [8] recently classified as *Tropicoporus linteus* (Berk. & M.A. Curtis) L.W. Zhou & Y.C. Dai. Polysaccharide fractions from the latter, particularly glucans, have effective therapeutic value confirmed in ongoing clinical trial with dosage up to 2 g/day for the duration of 8–10 weeks.[9] The safety of *P. linteus* as a non-toxic species was also established by the Chinese pharmacopeia, proposing a daily intake of 10–30 g dried mushroom or 2–3 g extracted polysaccharide.[10]

Furthermore, *Phellinus torulosus* (Pers.) Bourdot & Galzin, recently classified as *Fuscoporia torulosa* (Pers.) T. Wagner & M. Fisch., is a wood-decay fungus commonly found in Europe and North America acknowledged for its antioxidant and antimicrobial properties.[6,11] Another *Phellinus* species, *P. igniarius* (L.) Quél., also named *P. trivialis* (Bres.) Kreisel, is known as an edible basidiomycete, a saprotrophic fungus used in traditional medicine and recently identified as having high bioactivity, strong anticancer, antimicrobial and antioxidant effects highlighting its possible pharmacological applications.[12,13] Both species are non-toxic, and their antioxidant and therapeutic potentials could be used in modern medicine.

Enhanced potency of extracts *vs.* powdered form (pills), worthy of maximal solubility, absorptivity and bioavailability of β-glucans as one of the main antioxidants in *Phellinus* sp.,[14] as well as elimination of indigestible components contained in the powdered form made them interesting subject for analysis and popularization of their properties. The current work provides evidence of antioxidant potencies of *P. torulosus* and *P. igniarius* found on the Macedonian territory, intended for promoting their medicinal and health benefits.

2. EXPERIMENTAL

2.1. *Chemicals and reagents*

All of the reagents and solvents were of analytical grade (>98 % purity) including 2,2-diphenyl-1-picrylhydrazyl ($C_{18}H_{12}N_5O_6$, DPPH), trichloroacetic acid (CCl_3COOH, TCA), phosphate buffer solution (PBS), 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) ($C_{18}H_{18}N_4O_6S_4$, ABTS), Folin-Ciocalteu reagent (FCR), 6-hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid ($C_{14}H_{18}O_4$, Trolox), 1,10-phenanthroline ($C_{12}H_8N_2$); 2-*tert*-butyl-4-hydroxyanisole ($C_{11}H_{16}O_2$, BHA), sodium nitroprusside ($Na_2[Fe(CN)_5NO] \cdot 2H_2O$, SNP), *n*-butanol ($C_4H_9OH$, anhydrous, 99.8 %), 3,4,5-trihydroxybenzoic acid ($C_6H_2(OH)_3CO_2H$, Gallic acid), hydrochloric acid (HCl), 2-thiobarbituric acid ($C_4H_4N_2O_2S$, TBA), aluminum(III) chloride ($AlCl_3$), sulfuric acid (H_2SO_4), ammonium heptamolybdate tetrahydrate ($(NH_4)_6Mo_7O_{24}\cdot 4H_2O$),

bovine serum albumin (BSA), glucose ($C_6H_{12}O_6$), oxalic acid ($H_2C_2O_4$), 1,1,3,3-tetraethoxypropane ($C_7H_{16}O_4$, TEP) and methanol (CH_3OH, anhydrous, 99.8 %) which were purchased from Sigma Aldrich Co (St Louis, MO, USA). Ascorbic acid ($C_6H_8O_6$), iron(II) sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$), sodium carbonate (Na_2CO_3) and sodium phosphate (Na_3PO_4) were purchased from Merck. Commercial Griess reagent ($C_{18}H_{26}Cl_2N_3O_7PS$) and iron(III) chloride ($FeCl_3$) were obtained from Carlo Erba Chemicals.

2.2. *Collection of samples*

Mature fruiting bodies of *Phellinus torulosus* and *P. igniarius* (showing no physical damages and/or rotting signs) were collected from oak (*Quercus* sp.) trees at Taor Gorge in the vicinity of Skopje, RN Macedonia (coordinates: 41° 53' 57.04" N; 21° 36' 40.64" E). The lower part of the fruiting body was mashed to produce 10 g of each sample ($n = 3$) from each species. At least three fruiting bodies from each species were selected and subsequently used in the preparation of the extracts.

2.3. *Preparation of extracts*

As much as 10 g of each selected fresh fruiting body (part of the hymenophore) from both species ($n = 3$ per species) were grated in a blender and macerated in liquid nitrogen. The powdered samples were extracted using deionized water or methanol. Specifically, hot water extracts (HWEs) samples were extracted with 100 ml deionized water and boiled for 40 minutes at 100 ºC. After extraction, the mixtures were filtrated under cheesecloth, then passed through Whatman No 1 filter paper (Macherey-Nagel GmbH, Düren, Germany). Cold water extracts (CWEs) were obtained after dissolving the samples in 100 ml cold deionized water followed by 3 hours incubation in an orbital shaker. After the extraction, samples had the same treatment as HWE. Both extracts, HWEs and CWEs were additionally filtrated through 0.45 µm cellulose filter (Corning Inc., Corning, New York, USA) under vacuum. The obtained filtrates were used for subsequent analysis and measured in technical triplicates.[15] For methanol extraction, samples were mixed with 100 ml 60% (v/v) methanol and homogenized for 5 minutes with an ultrasonic homogenizer. The mixtures were held at +4 ºC for 24 hours. After the incubation, obtained supernatants were filtered through Whatman No 1 and used for further analyses.

2.3.1. *Nutritive status of the mushrooms*

The total protein, fat and ash content were assessed using fresh fruiting bodies ($n = 3$ per species) from *Phellinus torulosus* and *P. igniarius* mushrooms according to the methods developed by the Association of Official Agricultural Chemists (AOAC).[12] The data were expressed as g/100 g (%) fresh weight (FW). Polysaccharide content was determined in isolated polysaccharide fractions after ethanol precipitation with phenol-sulfuric assay[16] and presented in g/100 g (%) as defined by Deveci *et al.*[17]

2.3.2. *Content of bioactive compounds*

The phenolic compounds were determined by Folin-Ciocalteu method according to Ainsworth and Gillespie.[18] The optical density (OD) was read at 720 nm on double beam UV-visible spectrophotometer (Cary 60 UV-Vis, Agilent Technologies, United States). Total phenolic content was calculated against the calibration curve made by serial dilution of gallic acid (range of 0.1–0.0025 mg/ml) and the results were presented as milligram of gallic acid equivalents per gram (mg GAE/g) extract.

Total flavonoid content was determined by aluminum chloride method.[19] Absorbance was measured at 510 nm and the results were presented as mg of quercetin equivalents per gram (mg QE/g) extract.

2.3.3. *Free radical scavenging activity*

DPPH antiradical activity was determined according the method of Brand-Williams *et al.*[20] modified by Shori and Baba.[21] The decline in optical density (OD) was measured at 517 nm on the spectrophotometer using ascorbic acid as a positive control. Results were presented as percentage of inhibition calculated, with the blank sample treated in the same way.

Radical scavenging activity was determined by the method of decolorization of $ABTS^{\bullet+}$ with slight modifications.[22] Briefly, 20 µl sample was mixed with 2 ml ABTS reagent (a mixture of 2.45 mM potassium persulfate and 7 mM ABTS solution, stored in dark for 12–16 hours and properly diluted to an optical density (OD) of 0.70 ± 0.02 at 734 nm) and incubated for an additional 6 minutes in dark. Trolox was used as a positive control and the results were presented as percentage of inhibition.

Nitric oxide (NO) radical scavenging activity was assayed following the method adapted by Kumar and Kumar[23] with some variations. Specifically, 2 ml (10 mM) of sodium nitroprusside solu-

tion in phosphate buffer (pH = 7.4) was mixed with 1 ml fungal extract. After 120 minutes incubation at RT, 1 ml of the reaction mixture was collected and added to 1 ml of commercial Griess reagent. After 30 minutes' incubation period, optical density was read at 540 nm. Ascorbic acid was used as positive control and the results were expressed as percent of inhibition.

2.3.4. *Reductive capacity – phenanthroline method*

The method for determination of antioxidant capacity, described by Szydlowska-Czerniak *et al.*[24] was applied after slight modifications. The reaction mixture was incubated at RT for 20 minutes in dark conditions, and the OD was measured at 510 nm. The results were expressed as mM Fe(II), and calculated against the calibration curve of $FeSO_4$.

2.3.5. *Total antioxidant capacity – phosphomolybdenum method*

Total antioxidant capacity was estimated by the method of Prieto *et al.*[25] Briefly, 3 ml chromogen reagent containing sulfuric acid, sodium phosphate and ammonium heptamolybdate was added to the 0.3 ml of fungal extracts. The samples were incubated at 90 °C for 90 min. Absorbance was measured at 695 nm. Ascorbic acid was used as standard, and the results were presented as mg of ascorbic acid equivalents per gram (mg AAE/g) extract.

2.3.6. *Inhibition of lipid peroxidation (LPO)*

Assay of inhibition of lipid peroxidation was performed according to the modified procedure described by Ng *et al.*[26] In the 0.5 ml of freshly prepared 10% w/v mice liver homogenate (initiated with 0.1 ml 10 μM iron(II) sulfate and 0.1 ml (0.1 mM) ascorbic acid), 0.1 ml extract was added, and the mixture was incubated at 37 °C for 30 minutes. The reaction was stopped using 1 ml 20 % (w/v) TCA, followed by 1 ml 0.67 % (w/v) TBA. Samples were heated to 90 °C for 45 minutes. The lipid content was extracted into the *n*-butanol layer after centrifugation for 10 min at 3000 rpm. BHA was used as a positive control. The inhibition rate was calculated and presented as percent of the inhibition of lipid peroxidation process.

2.3.7. *Inhibition of protein glycation*

The assay was performed in BSA – Glucose medium according to Seri *et al.*[27] The formed glycation products were extracted with oxalic acid in boiling water bath, and centrifuged for 10 minutes at 3000 rpm. Supernatant was treated with 0.05 M TBA and incubated for 30 minutes at 40 °C. After incubation, OD was measured at 443 nm and the results were presented as inhibition percentage of protein glycation.

2.4. *Statistical analysis*

For each *Phellinus* species, three samples (three different fruiting bodies) were analyzed, each in triplicate. The results were presented as mean values ± standard deviation (SD). The results were evaluated using one-way repeated measures Analysis of Variance (ANOVA) where appropriate, operating the Statsoft STATISTICA 12 software. Duncan's multiple range test was performed for calculation of significant differences between the samples. Mean values were considered statistically significant when $p < 0.05$. Artwork was created in Graphpad Prism 6.0.

3. RESULTS AND DISCUSSION

3.1. *Nutritive content of the fungal extracts*

Results presented in Table 1, indicated that *P. torulosus* possesses significantly greater protein and fat content (16 % for protein and 66.7 % greater value for fat, respectively, $p < 0.05$), as well as richer mineral composition signified by the 74.3 % higher ash content compared to *P. igniarius* ($p < 0.05$), which was similar to the value registered by Ulziijargal and Mau[28] for the species *P. linteus*. However, both species had statistically close values for the polysaccharide content. In the study of Yang *et al.*[12] the reported values of ash and fat content in fruiting body of Taiwanese *P. igniarius* (values of 2.20 ± 0.04 % and 1.30 ± 0.07 %, correspondingly) were similar to those obtained in this study (2.10 ± 0.16 % and 1.59 ± 0.14 %), but with 22 % higher protein content than the *P. igniarius* from Macedonian origin. However, disparities in protein content might occur not only due to differences in environmental factors and the stage of maturity of the fruiting body, but also due to differences in type of measurements: carried out on fresh weight or on a dry samples, in which protein and fat content varies.[29,30] The above-mentioned authors reported an equivalent value for polysaccharide content. Measurements from other reports signify *Phellinus* spp. as a richer source of proteins compared to well-known edible mushrooms, such as *Agaricus bisporus* (J. E. Lange) Imbach (white button), *Lentinula edodes* (Berk.) Pegler (shiitake)

and *Flammulina filiformis* (Z. W. Ge, X. B. Liu & Zhu L. Yang) P. M. Wang, Y. C. Dai, E. Horak & Zhu L. Yang (enoki) (with protein range from 2.24 – 3.09 % FW)[31], *vs.* wild mushrooms belonging to *Auricularia* and *Russula* species (range between 3.16 and 5.19 % FW).[30]

T a b l e 1

Nutritive content of P. torulosus and P. igniarius

Species	Protein content (% FW)	Fat content (% FW)	Polysaccharide content (%)	Ash content (% FW)
P. torulosus	5.32 ± 0.05*	2.65 ± 0.07*	6.47 ± 0.86	3.66 ± 0.11*
P. igniarius	4.58 ± 0.08	1.59 ± 0.14	5.37 ± 0.67	2.10 ± 0.16

Results are presented as mean ± SD of three different fruiting bodies per species.
*Indicates statistical significance at $p < 0.05$ between both species.

3.2. *Bioactive compounds content*

Bioactive compounds such as polyphenols, polysaccharides and flavonoids isolated from medicinal fungi, can be useful in prevention/treatment against a number of diseases whose aetiology is connected with increased free radical formation, inflammation or malignant neoplastic processes.[32] From the results presented in Table 2, it can be observed that methanol extracts (MEs) from *P. torulosus* possessed two times higher quantity of phenolics *vs. P. igniarius* ($p < 0.05$). Furthermore, HWE from *P. igniarius* also contained richer bioactive potential compared to *P. torulosus* (42.8 % and 85.6 % higher amounts of phenolic and flavonoid compounds respectively, $p < 0.05$). Previously published results are comparable to ours regarding flavonoid content of MEs measured in *P. badius* (Cooke) G. Cunn., *P. gilvus* (Schwein.) Pat. and *P. rimosus* (26.49, 30.58 and 28.04 mg QE/g).[33] Similar values (0.95–8.84 mg GAE/g) for total phenolic content in 60 % MEs evidenced in wild edible mushrooms growing in Thailand, were also reported in the study of Butkhup *et al.*[34] CWEs from both species, presented lower levels of phenolic and flavonoid compounds. Overall, it can be concluded that enhancement of the extraction temperature and the polarity of the solvent is essential for obtaining high bioactive yield, so more polar mixtures such as aqueous methanol or ethanol are the most suitable extractants, where the optimum conditions for phenolic and flavonoid extraction are highly determined on the physicochemical composition of each species.[35] More recent research about the bioactive properties of *Phellinus* spp. published that a repetitive extraction of *P. vaninii* Ljub. (currently classified as *Sanghuangporus vaninii* (Ljub.) L.W. Zhou & Y.C. Dai) in methanol or hot water, provided significantly richer amounts of phenolic compounds compared to examined species in this study, which were extracted with a single type extraction.[36]

T a b l e 2

Bioactive content, reducing power (phenanthroline assay) and total antioxidant capacity of different extracts from Phellinus torulosus and P. igniarius

Specimens	Solvents	TP [mg GAE/g]	TF [mg QE/g]	Phen [mM Fe/g]	TAC [mg AAE/g]
Phellinus torulosus	HWE	$0.70 \pm 0.05^{a,A}$	$1.39 \pm 0.11^{a,A}$	$1.39 \pm 0.10^{a,A}$	$0.52 \pm 0.04^{a,A}$
	CWE	$0.16 \pm 0.04^{b,A}$	$0.09 \pm 0.06^{b,A}$	$1.05 \pm 0.19^{a,A}$	$0.28 \pm 0.02^{b,A}$
	ME	$10.94 \pm 0.71^{c,A}$	$20.35 \pm 0.79^{c,A}$	$4.92 \pm 0.68^{b,A}$	$3.92 \pm 0.34^{c,A}$
Phellinus igniarius	HWE	$1.00 \pm 0.08^{a,B}$	$2.58 \pm 0.11^{a,B}$	$0.92 \pm 0.20^{a,B}$	$0.63 \pm 0.05^{a,A}$
	CWE	$0.22 \pm 0.04^{b,A}$	$0.11 \pm 0.01^{b,A}$	$1.45 \pm 0.32^{a,A}$	$0.35 \pm 0.04^{b,A}$
	ME	$5.16 \pm 0.34^{c,B}$	$25.78 \pm 0.98^{c,B}$	$4.09 \pm 0.93^{c,A}$	$2.47 \pm 0.21^{c,B}$

Results are presented as mean ± SD of samples from fruiting bodies ($n = 3$) per species, measured in technical triplicates.
Different small letters within a column indicate statistical significance $p < 0.05$ between different extracts of the same species.
Different capital letters indicate statistical significance $p < 0.05$ between same extract from both species.

3.3. *Reductive power – phenanthroline assay*

The highest reducing power was detected in MEs ($p < 0.05$ *vs*. HWEs and CWEs, Table 2), in both species. The presence of valuable bioactive metabolites, such as polyphenols or flavonoids capable of donating a hydrogen atom and thus breaking the free radical formation, is essential for higher rates of transformation of ferric cation to ferrous.[37] However, close values were observed in CWEs and HWEs for both species which may be explained by the disparities between the phenolic and flavonoid content. Statistical significance was only observed between HWEs from the two species, with *Phellinus torulosus* having higher affinity for reduction of the ferric cation (51 % higher value than *P. igniarius*, $p < 0.05$). In comparison with the relatively limited results for the reductive properties of polypores or medicinal mushrooms in general, assayed by phenathroline protocol, HWEs from our samples were significantly lower than those obtained in HWEs from *Auricularia auricula-judae* (Bull.) Quél.[38] Moreover, the analyzed *Phellinus* species in this study showed greater reducing properties than the MEs from most of the tropical fruits, such as papaya, guava or mango.[39]

3.4. *Total antioxidant capacity – phosphomolybdenum assay*

The obtained values for the total antioxidant capacity in all extracts (Table 2) additionally confirmed that MEs had the higher antioxidant and reducing power, as evidenced by the reduction of the ammonium molybdate to molybdenum blue, due to their higher total phenolic and flavonoid content ($p < 0.05$ *vs*. HWEs and CWEs). Measurement of water extracts showed two times higher total antioxidant activity of HWEs *vs.* CWEs ($p < 0.05$).

Comparing the results between the two species, a statistical significance was only observed in MEs with 58.7 % higher value achieved for *P. torulosus* ($p < 0.05$) *vs. P. igniarius*, but both species revealed related antioxidant capacities for water extractant, independent of type of extraction (HWEs or CWEs). Previously reported results regarding HWEs from *Auricularia auricula-judae* demonstrated significantly higher antioxidant activity (7.6 ± 0.37 mg AAE/g) than the mushrooms examined in this study, presumably due to the higher extractability of phenolic acids (gallic acid and protocatechuic acid) in water extracts from *Auricularia* sp.[38]

3.5. *Radical scavenging activity*

Results presented in Table 3, from both species showed efficient DPPH and ABTS scavenging activities and richer bioactive content in methanol *vs*. water extracts, together with slightly higher ABTS inhibition rate in favor of *P. igniarius* (+12.8 %, $p < 0.05$). Similar findings regarding antiradical potency were observed in methanolic *vs.* aqueous extracts from *P. gilvus*.[11,37] Although HWEs from both species displayed equally potent DPPH scavenging activity, higher ABTS antiradical power was registered for *P. igniarius* (3 times greater ABTS neutralization rate, $p < 0.05$). It seems that in HWEs, additional scavengers (reducing sugars, α- and β-glucans) impact the antiradical activity of each extract.[40] The possible neutralizing effects of other compounds were observed in a study by Im *et al.*[36] who reported equal potency of HWEs and MEs from *P. vaninii* by achieving DPPH neutralization at 2 mg/ml, regardless of the significantly higher phenolic and flavonoid content detected in the ME of this fungus. Finally, this study found CWEs possessed minimum scavenging activity, in line with their lowest content of bioactive compounds.

Table 3

DPPH and ABTS scavenging activity of different extracts from Phellinus torulosus and P. igniarius

Specimens	Solvents	mg/ml	DPPH % inhibition	ABTS % inhibition
Phellinus torulosus	HWE	100	$59.77 \pm 1.58^{a,A}$	$12.64 \pm 0.75^{a,A}$
	CWE	100	$29.87 \pm 0.15^{b,A}$	$5.19 \pm 1.22^{b,A}$
	ME	10	30.34 ± 0.41^{A}	15.27 ± 0.33^{A}
Phellinus igniarius	HWE	100	$56.73 \pm 1.20^{a,A}$	$4.01 \pm 0.26^{a,B}$
	CWE	100	$33.18 \pm 0.01^{b,B}$	$1.92 \pm 0.34^{b,B}$
	ME	10	32.35 ± 0.18^{A}	$17.23 \pm 0.17^{\mathbf{B}}$

Results are presented as mean ± SD of samples from fruiting bodies ($n = 3$) per species, measured in technical triplicates. Different small letters indicate statistical significance $p < 0.05$ between different extracts of the same species. Different capital letters indicate statistical significance $p < 0.05$ between same extracts from both species.

From the results presented in Figure 1, it can be seen that NO scavenging activity increased in a dose-dependent manner in both fungal extracts, but again showing the highest scavenging activity in the MEs, pronouncing *P. igniarius* as a significantly stronger radical scavenger *vs. P. torulosus* (3 times higher neutralization rate at concentration of 1 mg/ml, correlated with its flavonoid content (Figure 1A).[41] According to previously published results, MEs from *Pleurotus floridanus* Singer were more efficient NO scavengers, with neutralization rate of ~80% at a concentration of 1 mg/ml.[42]

Hot water extracts indicated complete neutralization of NO radical at a higher concentration of 100 mg/ml for both species, confirming again *P. igniarius* superiority with two times higher scavenging rate (Fig. 1B, $p < 0.05$). However, both CWE and HWE from *P. torulosus* showed similar NO neutralization values, starting at 10 mg/ml (Figs. 1A and 1B).

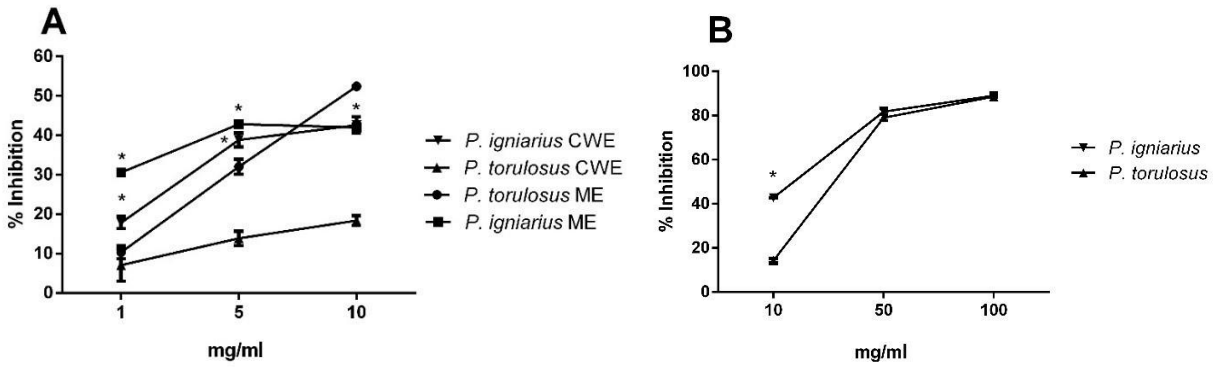

Fig. 1. NO scavenging activity of: A) Cold water and methanol extracts; B) Hot water extracts from *P. torulosus* and *P. igniarius.* Results are presented as mean ± SD of samples from fruiting bodies ($n = 3$) per species, measured in technical triplicates. * indicate statistical significance $p < 0.05$ between extracts at different concentration points.

3.6. *Inhibition of BSA glycation*

Bioactive compounds, mostly flavonoids, are the potent inhibitors of advanced glycation end products (AGEs) formation due to their interactions with proteins, even though the exact mechanism of their action still remains to be investigated.[43] Additional molecules such as polyphenols (caffeic and ellagic acid) or davallialactone, interfungin A and inoscavin A (detected in the ethyl-acetate fraction of *P. linteus*) were also added to the list of potential compounds that prevent protein glycation.[44] These findings point out the excellent antiglycation properties of medicinal mushrooms, which should be exploited in modern medicine. In our study, the highest rate of inhibition of *in vitro* induced protein glycation in BSA-glucose medium was achieved with 10 % CWE from both species (Figure 2), followed by MEs with significantly lower values (by 10.25 % and 8.3 % lower values compared to CWE for *P. torulosus* and *P. igniarius*, respectively, $p < 0.05$). The same percentage of HWE from *P. igniarius* showed the lowest antiglycation rate ($p < 0.05$). Evidence suggests that antiglycation effects are weakly correlated with the phenolic content,[45] since direct interactions of terpenoids and carotenoids with oxidative free radicals might also contribute to the reduction of the glycoxidation process.[46] In agreement with above, recent discoveries validate that fractions of crude CWE from *Lignosus rhinocerus* (Cooke) Ryvarden (tiger milk mushroom) possessed superior inhibition potential than each of the fractions with low, middle and high molecular weight, with positive correlation detected between the inhibitory rate and the antioxidant potential.[47]

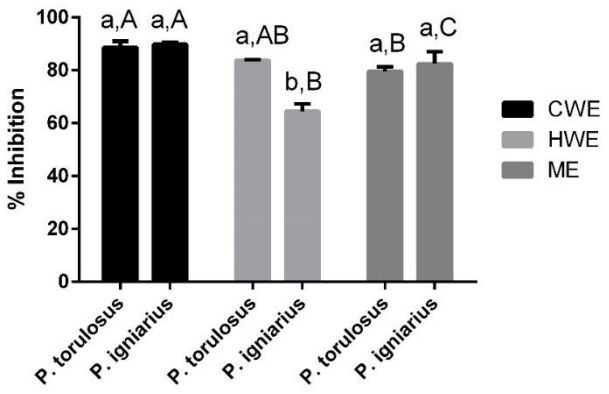

Fig. 2. Inhibition of BSA glycation of different extracts from *P. torulosus* and *P. igniarius* at concentration of 100 mg/ml (Results are presented as mean ± SD of samples from fruiting bodies ($n = 3$) per species, measured in technical triplicates). Different small letters indicate statistical significance $p < 0.05$ between different extracts of the same species. Different capital letters indicate statistical significance $p < 0.05$ between same extract from both species.

3.7. *Inhibition of lipid peroxidation (LPO)*

Measurement of inhibition of malondialdehyde (MDA) production, is a convenient marker for detection of the *in vitro* antioxidant potency of extracts.[47-49] In addition, lipid oxidation in foods and food products lowers their quality, creating off-flavors and unhealthy compounds. Therefore, it is of great interest to prevent reactive oxygen species (ROS) production and lipid oxidation, all in order to improve the quality of processed foods. From the results presented in Figure 3, it is obvious that the inhibition of the formation of thiobarbituric acid adducts (TBARs) increased in a dose-dependent manner of the added extract, with the highest rates observed for MEs and CWEs in both species (inhibition rate within the range of 90–94 % at a concentration of 100 mg/ml). The lowest effects on TBARs synthesis was detected in HWEs from both species with inhibition of 72–73 % at the concentration of extract of 100 mg/ml ($p < 0.05$). This method has been examined in methanolic extracts from wild medicinal mushrooms like *Antrodia camphorata* (M. Zang & C. H. Su) Sheng H. Wu, Ryvarden & T. T. Chang (currently classified as *Taiwanofungus camphoratus* (M. Zang & C. H. Su) Sheng H. Wu, Z. H. Yu, Y. C. Dai & C. H. Su) which showed similar results to ours (5.32–5.78 % of inhibition at 1 mg/ml). Nevertheless, several other species such as *Grifola frondosa* (Dicks.) Gray (maitake) and *Hericium erinaceus* (Bull.) Pers. (lion's mane), showed significantly higher inhibitory activities (29.81 % and 48.45 %, respectively) at a much lower concentration of 1.2 mg/ml.[4] Other studies have confirmed the lower scavenging ability of TBA-MDA adducts of HWEs *vs*. MEs, established by the assessments obtained for 14 edible medicinal mushrooms ranging from 33.33 % to 57.18 % at concentration of 10 mg/ml, which are comparable with our results about *Phellinus* species.[50]

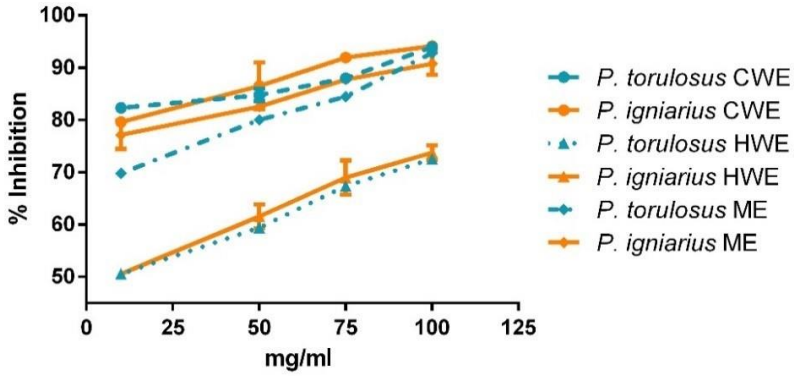

Fig. 3. Effects of different extracts from *P. torulosus* and *P. igniarius* on LPO inhibition rate.
Results are presented as mean ± SD of samples from fruiting bodies ($n = 3$) per species, measured in technical triplicates

4. CONCLUSION

From the comparative analysis of the bioactive potentials and *in vitro* radical scavenging abilities of three different extracts from *Phellinus* spp., it can be concluded that methanol is a more suitable solvent because it obtained significantly higher phenolic and flavonoid yields which were directly proportional to the inhibition rates of radicals DPPH, ABTS, and NO, as well as inhibition of lipid peroxide formation (LPO inhibition). Hot water extraction influenced better antioxidant activity than CWEs with *P. igniarius* showing significantly higher effects than *P. torulosus*, even though their bioactive contents seemed to moderately correlate with the radical scavenging abilities. Cold water extractions were superior in antiglycation properties. Further clarification of the correlation between fungal composition, area of distribution and the antioxidant activities of *Phellinus* species across Macedonian territory will be the subject of further investigation for their possible use as medicinal remedies.

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