Bioactive Compounds, Antioxidant and Anti-inflammatory Activities of Extracts from *Cantharellus cibarius*

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Cantharellus cibarius is an edible mushroom found in the wild, which is widely used in alimentation due to its nutritive composition and special taste. The antioxidant properties of ethanolic, methanolic, hot water and cold water extracts were performed using in vitro (DPPH radical scavenging activity, reducing power, metal chelating ability, inhibition of lipid peroxidation, superoxide, and hydroxyl radicals scavenging ability) and in vivo methods (with yeast cells). Anti-inflammatory activity was proven by inhibition of albumin denaturation, erythrocyte membrane stabilization and proteinase inhibitory activity. Major phenolic compounds were homogentisic acid and gallic acid that were determined by HPLC methods. The results showed that all extracts exhibited antioxidant and anti-inflammatory activities. A significant correlation was found between antioxidant and anti-inflammatory activities and the antioxidant components, especially major phenolics and carotenoidic compound (lycopene).

Keywords: golden chanterelle, scavenging activity, anti-inflammatory activity, survival capacity, homogentisic acid

Cantharellus cibarius, found in Romania, also known as golden chanterelle, is an edible mushroom highly sought-after due to its gastronomic qualities. It is found in abundance in the coniferous forests, and is harvested during summer and in early autumn. Also, it can be purchased in desiccated form in food stores, and is in great demand due to its special flavor and the slightly spicy taste [1].

Wild edible mushrooms represent a food product, but are also one of the natural sources of physiologically active compounds used in native medicines [2]. These species of mushrooms have a variety of metabolites, such as phenolic compounds, terpenes, and steroids. The mushrooms contain lectins, various types of polysaccharides, and proteins well known for their immunomodulatory and antiproliferative activities against cancerous cells [3]. Besides these properties, anti-inflammatory, antibacterial, and even antiviral effects have been demonstrated. The main biomolecules which exercise the most important biological effects are the phenolic compounds, flavonoids, and tannins [4].

There are a few studies concerning the analysis of the nutraceutical composition of the mushroom species *C. cibarius*. Such studies have indicated that the mushroom has a nutraceutical composition comparable with other renowned varieties of mushrooms, combined with a significant antioxidant activity [5]. For the mushrooms harvested in Romania, there are no such studies, although the mushroom is extensively harvested for export. For a full assessment of the mushroom, the scavenging capacity of the radical DPPH, the reducing power, inhibition of lipid peroxidation, iron chelating capacity, superoxide, and hydroxyl scavenging activity were determined. An *in vitro* assessment of the anti-inflammatory capacity was also performed. The *in vivo* assessment of the antioxidant activity was performed with two yeast strains (*Saccharomyces cerevisiae* and *Pichia pastoris*). The contents of the antioxidant components were also determined by means of chromatography.

Experimental part

Materials and materials

Dried fruit bodies of *C. cibarius* were obtained from a supermarket in Bucharest, Romania. All chemicals and reagents were purchased from Sigma, Aldrich GmbH, Sternheim, Germany. All other unlabeled chemicals and reagents were of analytical grade.

Extraction. Dried samples were subject to extraction with 70% ethanol, 70% methanol, hot water, and cold water. A 10 g sample of the dried mushrooms was extracted using 100 mL solvent (ethanol and methanol) for 24 h, at 25 °C; 10 g of sample was boiled in 500 mL of water for 30 min. For cold water extraction, a sample (10 g) was extracted by stirring with 100 mL cold water at 4 °C for 24 h [2]. The extract was filtered using a Whatman No. 1 filter paper [3]. The solvents used for extraction were removed using a Buchi R215 rotary vacuum evaporator, with the vacuum controller V-850 and a Multivapor P-6 heating bath for parallel evaporation at 50 °C under vacuum [6,7]. The resulting extracts were lyophilized in an ALPHA 1-2 LD plus freeze dryer (Martin Christ GmbH Gefriertrocknungsanlagen), at –55°C, for 48 h. The *reconstitution solvent* consisted of 80% ethanol and the sample quantity range was between 2 – 10 mg/mL.

Determination of in-vitro antioxidant activities

DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. A quantity consisting of 0.8 mL of 0.2 mM DPPH solution was mixed with a 0.2 mL different concentration of the extracts (2-10 mg/mL). The mixture was shaken and left to stand for 30 min. The absorbance was measured at 517 nm using a Helios λ spectrophotometer. The DPPH radical scavenging activity (%) was calculated with the

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following equation: 1 – (As/Ac) ×100, where As is the absorbance in the presence of sample and Ac is the absorbance in the absence of sample. Ascorbic acid was used as standard. The EC_{50} value (mg extract/mL), being the amount of sample required to make 50% change in absorbance [6,8].

Reducing power. Each extract (in 2.5 mL of ethanol) was mixed with 2.5 mL of 200 mM sodium phosphate buffer (*p*H 6.6) and 2.5 mL of 1% potassium ferricyanide, and the mixture was incubated at 50°C for 20 min. Next, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged at 3000 g for 10 min. The upper layer (2.5 mL) was mixed with 2.5 mL of deionized water and 0.5 mL of 0.1% ferric chloride. Finally, the absorbance was measured at 700 nm. The extract concentration providing 0.5 of absorbance (EC_{50}) was calculated from the graph of absorbance at 700 nm plotted against the extract concentration. Ascorbic acid was used as positive controls [6, 9 - 11].

Determination of hydroxyl radical scavenging activity

Quantities consisting of 0.2 mL of 0.1 mM $FeSO_4$/0.1 mM EDTA·2Na, 0.2 mL 2-deoxyribose (10 mM), 0.2 mL sample (different concentration 2–10 mg/mL), and 1.2 mL phosphate buffer (0.1 M; *p*H 7.4) were mixed. After the addition of 0.2 mL H_2O_2 (10 mM), the mixture was incubated at 37 °C for 4 h, and the reaction stopped by addition of a 1 mL trichloroacetic acid (2.8%) solution. Thiobarbituric acid 50 mM in NaOH 1% (1 mL) was then added and the mixtures heated at 100 °C for 10 min, followed by rapid cooling and measurement of OD_{532} [6,12].

Nitric Oxide Scavenging of Freeze-Dried Extracts

Nitric oxide scavenging activity was measured spectrophotometrically. Sodium nitroprusside (5 mmol/L) in phosphate buffered saline, *p*H 7.4, was mixed with different concentrations of the extract (different concentration 2–10 mg/mL) prepared in ethanol 80% and incubated at 25 °C for 30 min. A control without the test compound, but with an equivalent amount of ethanol, was also used. After 30 min, 1.5 mL of the incubated solution was removed and diluted with 1.5 mL of Griess reagent (1% sulphanilamide, 2% phosphoric acid and 0.1% N-1-naphthylethylenediamine dihydrochloride). Absorbance formed during diazotization of the nitrite with sulphanilamide and subsequent coupling with N-1-naphthylethylene-diamine dihydrochloride was measured at 546 nm and the percentage scavenging activity was measured with reference to the standard (ascorbic acid). The EC_{50} value (mg extract/mL), being the amount of sample required to make 50% change in absorbance [6,13].

Ferrous ion Chelating Assay

1 mL of the sample (2–10 mg/mL) was mixed with 3.7 mL of ultrapure water, following which the mixture was reacted with ferrous chloride (2 mmol/L, 0.1 mL) and ferrozine (5 mmol/L, 0.2 mL) for 20 min. The absorbance at 562 nm was determined spectrophotometrically. EDTA was used as positive control. The chelating activity on the ferrous ion was calculated using the equation below: Chelating Activity (%) = [(Ab-As)/Ab]×100, where Ab is the absorbance of the blank without the extract or ascorbic acid and As is the absorbance in the presence of the extract or ascorbic acid [6,14].

Inhibition of lipid peroxidation

The reaction mixture contained 1mL of fowl egg yolk emulsified with phosphate buffer (*p*H 7.4) to obtain a final concentration of 25 g/L, sample (different concentration 2–10 mg/mL), and 100µL of 1000µM $FeCl_2$. The mixture was incubated at 37°C for 1 h before being treated with 0.5mL of freshly prepared 15% trichloroacetic acid (TCA) and 1.0mL of 1% thiobarbituric acid (TBA). The reaction tubes were further incubated in boiling water bath for 10 min. Once cooled to room temperature, the tubes were centrifuged at 3500 g for 10 min to remove precipitated protein. The absorbance at 532 nm was determined spectrophotometrically. Ascorbic acid was used as positive control. The percentage inhibition was calculated from the following equation: Inhibition (%) = [(Ab-As)/Ab]×100, where Ab is the absorbance of the blank without the extract or ascorbic acid and As is the absorbance in the presence of the extract or ascorbic acid [6,14,15].

Determination of the antioxidant activity in vivo

We used two yeast strains from the collection of the Faculty of Biotechnologies: *S. cerevisiae* R-BF and *P. pastoris* E7FB. The two strains were cultivated in YPG medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose), 24 h, 150 rpm. The obtained cells were separated by centrifugation (Hettich 320R) at 3000 rpm, for 10 min. They were washed twice with NaCl 0.9% and then resuspended in the same sterile solution to a final concentration of 1×10^6 viable cells/mL. In 5 sterile Eppendorf tubes (1.5 mL), 0.5 mL of cell suspension was introduced, with different volumes from the stock solutions of extracts (50 mg/mL) in order to treat the cells with concentrations of 2, 4, 6, 8, and 10 mg/mL and hydrogen peroxide 4 mM. The tubes were introduced in a Memmert thermostat for 1 h at 28–30 °C. Finally, the samples were centrifuged and diluted with NaCl 0.9%, and were seeded in Petri dishes with YPG + 20 g/L agar. After 48 h, the viability to ColonyQuant was read with the help of a corresponding software, by comparison with a sample of untreated cells [16, 17].

In vitro anti-inflammatory activity

Inhibition of albumin denaturation.The reaction mixture consisted of test extracts (2–10 mg/mL) and 1% solution of bovine albumin. The mixture were incubated at 37 °C for 20 min and then heated to 51 °C for 20 min. After cooling the samples, the turbidity was measured at 660 nm. Inhibition of protein denaturation (%) = $[(A_{control} - A_{sample})/A_{control}] \times 100$, where $A_{control}$ is the absorbance of the control, and A_{sample} is the absorbance of the sample extract/standard [18].

Erythrocyte membrane stabilization. Fresh human blood from the first author was collected and transferred to centrifuge tubes. The tubes were centrifuged at 3000 rpm for 10 min and were washed 3 times (in NaCl 0.9%). The blood cells were resumed in 10% saline solution. The reaction mixture (2 mL) consisted of 1 mL of sample solution (2–10 mg/mL) and 1 mL of 10% blood cells suspension, and was incubated at 56 °C for 30 min. At the end of the incubation, the tubes were cooled. The tubes was centrifuged at 2500 rpm for 5 min, and the absorbance of the supernatants was taken at 560 nm. Percent erythrocyte stabilization activity was calculated by the formula mentioned above [18].

Proteinase inhibitory activity

The reaction mixture (2 mL) contained 0.06 mg trypsin, 1 mL of 20 mM Tris HCl buffer (*p*H 7.4), and 1 mL test sample (2–10 mg/mL). The reaction mixture was incubated at 37 °C for 5 min, after which 1 mL of 0.8% casein was added. The mixture was incubated for an additional 20 min, and 2 mL of 70% perchloric acid was

added. The cloudy suspension was centrifuged, and the absorbance of the supernatant was read at 210 nm. Percent proteinase inhibitory activity was calculated using the formula mentioned above [18].

Determination of Antioxidant Component

Determination of Total Phenolic. Content. The content of total phenols was determined by spectrophotometry, using gallic acid as standard, according to the method described by the International Organization for Standardization (ISO) 14502-1. Briefly, an aliquot of the diluted sample extract (1.0 mL) was transferred in duplicate to separate tubes containing a 1/10 dilution of Folin-Ciocalteu's reagent in water (5.0 mL). Then, a sodium carbonate solution (4.0 mL, 7.5% w/v) was added. The tubes were then allowed to stand at room temperature for 60 min before absorbance at 765 nm was measured against water. The content of total phenols was expressed as gallic acid equivalents in g/100 g extract. The concentration of polyphenols in samples was derived from a standard curve of gallic acid ranging from 10 to 50 g/mL (Pearson's correlation coefficient: $r^2 = 0.9996$) [19,20].

Determination of Total Flavonoids. Sample (0.25 mL of different concentrations of the extracts) was added to a tube containing distilled water (1 mL). Next, 5% $NaNO_2$ (0.075 mL), 10% $AlCl_3$ (0.075 mL) and 1 M NaOH (0.5 mL) were added sequentially at 0.5 and 6 min. Finally, the volume of the reacting solution was adjusted to 2.5 mL with double-distilled water. The absorbance of the solution at a wavelength of 410 nm was detected using the Helios λ spectrophotometers. Quercetin a ubiquitous flavonoid, present in many natural extracts, used as standard to quantify the total flavonoid content. Results were expressed in microgram quercetin equivalents/100 g extract [20, 21, 22].

Determination of β-carotene, and lycopene. *F*or β-carotene and lycopene determination, the dried ethanolic extract (100 mg) was vigorously shaken with an acetone–hexane mixture (4:6, 10 mL) for 1 min and filtered through Whatman No. 1 filter paper. The absorbance of the filtrate was measured at 453, 505, and 663 nm. β-Carotene and lycopene content were calculated according to the following equations: lycopene (mg/100 mL) = $-0.0458 \times A_{663} + 0.372 \times A_{505} - 0.0806 \times A_{453}$; β-carotene (mg/100 mL) = $0.216 \times A_{663} - 0.304 \times A_{505} + 0.452 \times A_{453}$.The results are expressed as mg of carotenoid/g of extract [5,20].

Determination of ascorbic acid. Determination of ascorbic acid was determined by the method described by Barros et al. (2008) [5]. Content of ascorbic acid was calculated on the basis of the calibration curve of L-ascorbic acid, and the results were expressed as mg of ascorbic acid/100 g extract [23].

Determination of the total quantity of antocyanidins.The following solutions were used: ethyl alcohol, hydrochloric acid, sol.1.5 M, and cyanidin chloride, whereby the solvent was a mixture of 85:15 (v/v) of ethyl alcohol and hydrochloric acid 1.5 M. In a measuring bottle of 10 mL, 0.5 g of sample to be analyzed was weighed. Next, the solvent was added up to the mark, and ultrasonation for 15 min was performed. Filtering, with the help of Watman filter paper No. 1, was carried out. As standard solution, the cyanidin chloride with a concentration of 5–15 μg/mL, in solvent was used. The absorption rates of the sample and standard solutions, at spectrophotometer at 546 nm, was determined. The total quantity of antocyanidins (expressed in g of cyanidin chloride/100 g extract) = $(A_p \times m_{st} \times f \times 100)/(A_{st} \times m_p)$, where: A_p = absorption rate of the sample solution; m_p = mass of the processed sample, in g; A_{st} = absorption rate of the standard solution; m_{st} = mass of the processed standard solution, in g; f = dilution coefficient [6].

Determination of polyphenol carboxylic acids and flavones. Reagents and equipment: methanol HPLC, orthophosphoric acid, ultrapure water, absolute ethanol, high-pressure liquid chromatograph (HPLC) ELITE – LaChrom, with DAD detector, analytic scales KERN 770, solvent:ethanol – water (50:50, V/V). Sample solution: In a measuring bottle of 50 mL, we introduced 0.5000 g of sample powder, adding around 40 mL solvent, and performing ultrasonation for 30 min at 40°C. Supplements to 50 mL with the same solvent and filters were added as follows. Reference solution was as follows; mixture of 10 μg/mL: chlorogenic acid, coffee acid, cumaric acid, ferulic acid, lutheoline 7-glycoside, rutin, rosemary acid, apigenin 7-glycoside, lutheoline, apigenin, quercetin, kaempferol, catechin, myricetin, pyrogallol [6].

Chromatographic working conditions. The chromatographic column from stainless steel, comprised the stationary stage of octadecylsylane, (Inertsil ODS-3 250 . 4.6 mm . 5 μm); mobile stage: mobile stage A: phosphoric acid/water, *p*H = 2.5; mobile stage B: methanol; flow rate of the mobile stage: 1.0 mL/min; elution type: with linear composition gradient of the mobile stage; UV detection:λ = 330 nm; temperature of column oven: 40°C; injection volume: 20 μl. After the chromatographic system was balanced, the basic line is a straight line, and the reference solution is injected. The differences between successive determinations must be at a maximum of 2%. We injected the test solutions and registered the chromatograms. The content in the compounds of interest was calculated using the formula: compound % = $[(A_p \times C_e) \times A_e] \times (50/G) \times 100$, where: A_p: range of the compound's peak "i" in sample solution; A_e: range of the compound's peak "i" in the reference solution; C_e: concentration of compound "i" in the reference solution (mg/mL); G: quantity of processed sample, (mg); 50: correction coefficient [6].

Determination of tocopherols. Reagents and equipment. Ultrapure water; 2-propanol (R); acetonitrile (HPLC); tetrahydrofuran (R); high-pressure liquid chromatograph (HPLC) ELITE – LaChrom, with DAD detector; analytic scales KERN 770.

Working solutions. In a measuring bottle of 20 mL, exactly 15 mg of sample powder was weighed, adding 10 mL of water and performing ultrasonation for 15 min. Supplements to 20 mL with absolute ethyl alcohol, and performing ultrasonation for 5 min was carried out. Reference solution: In a measuring bottle of 25 mL, exactly 15 mg of tocopherol was weighed and dissolved in 10 mL of absolute ethyl alcohol and supplements up to the mark with the same solvent; then 2 mL of this solution was diluted at 10 mL, in a measuring bottle with the same solvent [6].

Chromatographic working conditions. The chromatographic column from stainless steel comprised the stationary stage octadecylsylane (ZORBAX Elipse XDB-C8

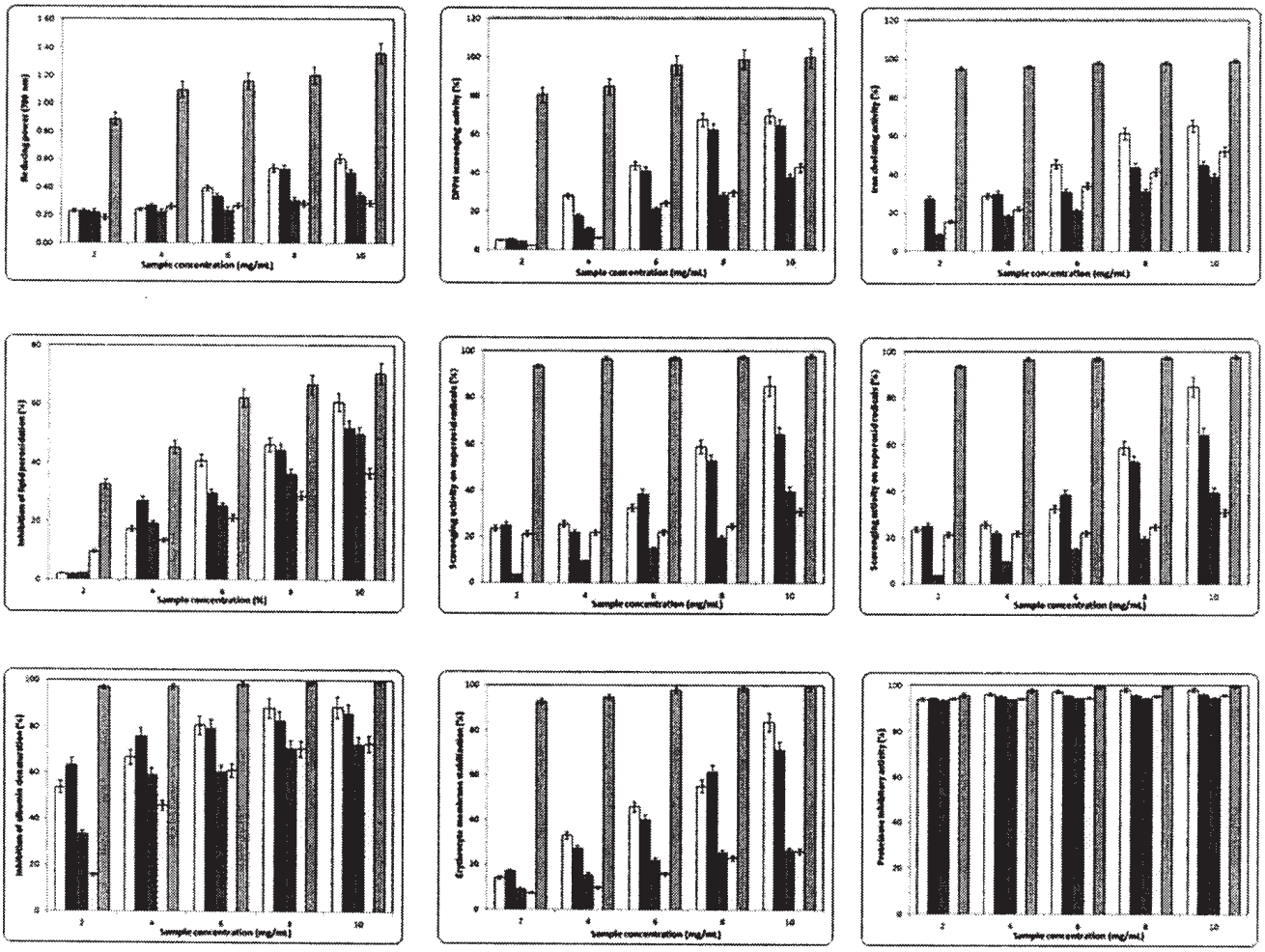

Fig. 1 Biochemical characteristics of different solvent extracts from *Cantharellus cibarius*.
□ Ethanol ■ Methanol ▩ Hot water □ Cold water ▨ Standard Each value is expressed as mean ± standard deviation (n = 3).
The results were significantly different $p < 0.05$

250 . 4.6 mm . 5 μm); mobile stage: methanol:water (95:5), V/V; flow rate of the mobile stage: 1.2 mL/min; detective: 280 nm; injection volume: 20 μL. After the chromatographic system was balanced, the basic line was a straight line, and the reference solution was injected. The differences between successive determinations must be at a maximum of 2%. We injected the test solutions and registered the chromatograms. The content in the compounds of interest was calculated using the formula: tocopherol (%) = $[(A_p \times C_e) \times A_e] \times (25/G) \times 100$, where, A_p: range of the tocopherol peak in the sample solution; A_e: range of the tocopherol peak in the reference solution; C_e: tocopherol concentration in the reference solution (mg/mL); G: quantity of processed sample, (mg); and 100 is the correction coefficient [6].

Statistical analysis

All parameters for antioxidant activity were assessed in triplicate, and the results were expressed as mean ± SD values of 3 observations. The mean values and standard deviation were calculated with the EXCEL program from Microsoft Office 2010 package. Statistical analysis was carried out using GraphPad Prism 6.0. Significant level was set at $p \leq 0.05$.

Results and discussions

Extraction Yield

Alcoholic extracts displayed the highest extraction yields from *C. cibarius*. Such conflict with other studies indicated that hot water extracts from *Coprinus comatus* have high values due to the presence of water-soluble components, for example, polysaccharides [24]. The order of succession of the extraction yields was: methanol (19.24% ± 0.22%) > ethanol (18.38% ± 0.36%) ≈ hot water (18.36% ± 0.31%) > cold water (15.7% ± 0.27%) ($p<0.04$).

Reducing Power

Reducing power is an important indicator of antioxidant activity, which proves that a direct relationship between both of them does exist [25]. The values determined represent an indicator of the capacity to donate electrons displayed by biomolecules with antioxidant effect [26]. Depending on the method used, reduction of F^{3+} to Fe^{2+} takes place. The reducing power of the extracts from *C. cibarius* increases pro rata with the concentration thereof (fig. 1). The colour of the solution changes from yellow to different shades of green, depending on the concentration of the sample. Depending on the solvent, the reducing power varied between 0.18 ± 0.02 and 0.6 ± 0.05, at a maximum concentration of 10 mg/mL. It was determined that alcoholic extracts displayed higher values of the reducing power than those in hot and cold water. As compared with the maximum reducing power obtained (the ethanolic extract), the ascorbic acid displayed a value 44.48% ± 0.16% higher, of 1.36% ± 0.24% ($p<0.05$).

Scavenging Ability on DPPH Radicals

The use of the radical, DPPH, in assessing the antioxidant capacity of an extract, is currently the most widely adopted method. Its use is favored due to its stability. The tested extract, by reacting with the substrate, and due to the compounds with antioxidant effect, may donate

hydrogen atoms. The DPPH solution which is violet loses colour until it becomes straw yellow, depending on the quantity of antioxidant compounds [27]. The data obtained (fig. 1) indicates that the extracts underwent a DPPH scavenging activity between 65.38% and 38.48%, at a concentration of 10 mg/mL ($p<0.02$). The succession order of extracts was: ethanol > methanol > cold water > hot water. At the same concentration, the ascorbic acid reached the value of 99% for DPPH scavenging activity. Therefore, the DPPH scavenging effect of *C. cibarius* extracts increases pro rata with the concentration. The data correspond to previous studies [28], which confirm that the methanolic extract had, for the maximum concentration, a scavenging activity of approximately 60%. In exchange, the current study indicated that the ethanolic extract had a ≈ 7% higher value of 69.45%.

Metal chelating activity

Ferrous iron can initiate lipid peroxidation by the Fenton reaction, as well as accelerating peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals. Ferrozine can quantitatively form complexes with Fe^{2+} [29]. In the presence of other chelating agents, the complex formation is disrupted with the result that the red-pink color of the complexes decreases. Measurement of colour reduction, therefore, allows the estimation of its metal chelating ability [30]. Thus, it is considered that the chelating abilities of the natural extracts correspond mainly to the content of phenols. For a concentration between 2 and 10 mg/mL, the metal chelating ability was between 21.92 and 65.38% for ethanolic extract, 27.05 and 44.61% for methanolic extract, 8.46 and 38.48% for hot water extract, and 15.38 and 51.92% for cold water extract (fig. 1) ($p<0.05$). The chelating ability of the extracts and the standard (EDTA) depends on the concentration of the sample. The data obtained represent the first determination of the chelating properties for lyophilized extracts from the wild edible mushroom *C. cibarius*. The values are comparable with those obtained for *Ganoderma tsugae* and *Agrocybe cylindracea (*42.6% and 45.8%), respectively, with those of hydrous extracts [9]. In exchange, the ethanolic extract displays a ≈18% more reduced activity than the hydrous extract from *Pleurotus squarrosulus*, for a ≈10% smaller concentration.

Lipid peroxidation

In biological systems, lipid peroxidation generates a number of degradation products, and is the primary cause of cell membrane destruction and cell damage [31]. It is considered that lipid peroxidation is a major cause for cardiovascular diseases and cancer. The inhibition capacity of an extract represents a direct indicator of the therapeutic potential [32]. The effect of *C. cibarius* extracts on inhibition of lipid peroxidation is presented in figure 4. At the maximum concentration of 10 mg/mL, the values obtained varied between 36.53% ± 0.4 and 60.57% ± 0.31% ($p<0.05$). The ascorbic acid had a ≈14% higher value than the maximum value determined for the ethanolic extract. Also, in the case of lipid peroxidation, the ethanolic and methanolic extracts displayed values higher than the hot and cold water extracts. Inhibition on lipid peroxidation increased pro rata with the concentration of the sample (extract) in all cases. The alcoholic extracts from *C. cibarius* had higher percentages than hot water extracts from *Ganoderma lucidum* (57.18%) and *Pleurotus florida* (56.58%), thus proving the higher extraction power of alcohols [15]. In exchange, the methanolic extract from *Leucopaxillus giganteus* cultivated in the presence of $(NH_4)_2HPO_4$ displayed ≈14.5% higher values as compared with the ethanolic extract, and 26.7% higher values as compared with the methanolic extract [33].

Scavenging activity on superoxide radicals

The superoxide anion radical is not very reactive, but its generation favours the production of hydrogen peroxide and of hydroxyl radicals. At the cellular level, the damages are determined by its derivatives, but not directly [34]. The scavenging activity on superoxide radicals ranged between 30.95% ± 0.23% and 84.82% ± 0.64%, for a maximum concentration of 10 mg/mL. The scavenging ability of ascorbic acid (as standard) 10 mg/mL, was 13.13% higher as compared with the maximum obtained value of extracts from *C. cibarius* ($p < 0.01$). Consequently, the succession order of extracts, depending on the maximum obtained value was, ethanolic extract > methanolic extract > hot water extract > cold water extract (fig. 1). By comparison with the alcoholic and hydrous extracts from the stipe and cap of *C. comatus*, the results are ≈ 30% higher for the alcoholic extracts, and ≈10% higher for the hydrous extracts [23]. Also, the values of the alcoholic extracts are comparable with those of *Armillaria mellea*, whose mycelium was cultivated submerged [35].

Scavenging activity on hydroxyl radicals

The hydroxyl radical is the most reactive of the reactive oxygen species, and it induces severe damage in adjacent biomolecules. The hydroxyl radical can cause oxidative damage to DNA, lipids, and proteins [20,36]. Thus, eliminating the hydroxyl radical is vital for the protection of living systems against various diseases [37]. The hydroxyl radical-scavenging activities of the various extracts from *C. cibarius* are presented in figure 1, increasing in a dose-dependent way [38]. At a concentration of 10 mg/mL, the ethanolic extract exhibited 67.6% scavenging activity on hydroxyl radicals. The scavenging effect (at the same concentration) of *C. cibarius* extracts and standard (ascorbic acid), decreased in the order: ascorbic acid > ethanol extract > methanol extract > hot water extract > cold water extract. However, there was statistically a very significant correlation between the values of the extracts and control ($p<0.05$) [39].

In vivo antioxidant activity

The purpose of this test is to observe the correlation of the antioxidant potential determined *in vitro* as compared with that determined *in vivo* [16,17]. The action of hydrogen peroxide on yeast cells is manifested by means of the hydroxyl radicals that act selectively on the cell membrane, thus causing its deterioration [40]. It was noticed that the alcoholic and hydrous extracts from *C. cibarius* increase the survival capacity after being treated with H_2O_2. Thus, the keeping of viability is directly proportional with the increase in the concentration of the extract. On average, the viability difference amounted to approximately 18% for *S. cerevisiae* R-BF, and 6% for *P. pastoris* E7FB. The presence of the extract had, as effect, the keeping of a part of the viability as compared with the cells which interacted with the hydrogen peroxide (fig. 2). The differences between the two strains were more and more obvious while increasing the concentration of the tested extract. The protecting capacity of the extracts followed the order: methanolic extract > hot water extract > cold water extract. For the two hydrous extracts, the difference, as compared with the control sample, did not exceed 10% (data not shown).

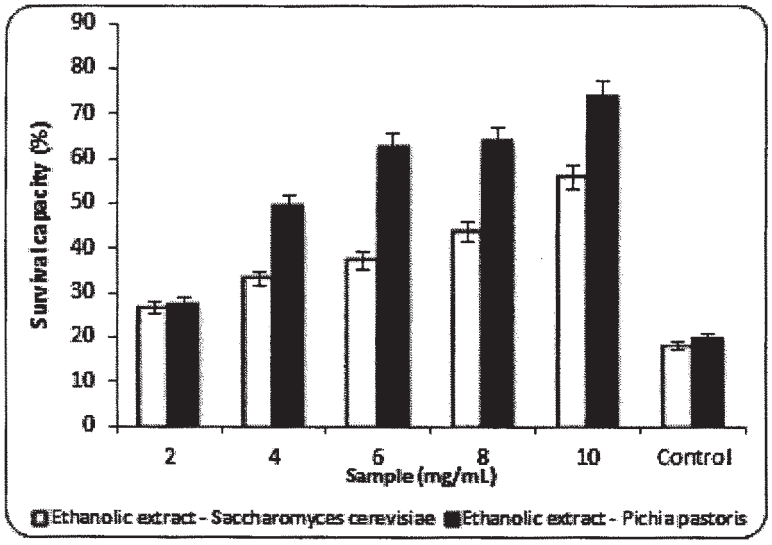

Fig. 2 Survival capacity of yeast cells after treatment with ethanolic extract from *Cantharellus cibarius*. Each value is expressed as mean ± standard deviation (n = 3). The results were significantly different $p < 0.05$

Concerning the correlation between the antioxidant capacity *in vitro* and *in vivo*, a significant linear relationship was calculated by reference to free radicals scavenging activity (R^2 = 0.8053 – 0.8445), the reducing power (R^2 = 0.7658 – 0.8935), the chelating capacity (R^2 = 0.7906 – 0.8444), the inhibition of lipid peroxidation (R^2 = 0.8913 – 0.9482). The prior presented values correspond to the ethanolic extract ($p<0.005$), while the other extracts had a good correlation (methanolic extract) and a weak one in the case of cold or hot water extracts (R^2 <0.56). The results prove the importance of assessing the relationship between the active mechanisms of the antioxidant effect [41,42].

In vitro anti-inflammatory activity

The cellular components most susceptible to damage by free radicals are lipids (by peroxidation process), proteins (by denaturation process), and nucleic acids (disturbing normal cell cycle). Denaturation of proteins is a major cause of inflammation, resulting in the onset of rheumatoid arthritis. Denaturation of proteins is a process during which they lose their tertiary and secondary structure by applying an external stimulus [43]. This process was presented for the four extracts by determining the inhibiting albumin denaturation. Maximum inhibition (10 mg/mL sample concentration) was 88.32% ± 1.67% for ethanol extract, followed by methanol (85.58% ± 2.43%), cold water (72.64% ± 1.8%), and hot water (61.39% ± 1.01%) extracts (fig. 1). The minimum difference, as compared with the standard (diclofenac sodium) was 11% ± 0.9% ($p<0.05$). An increase of the inhibition capacity (%) in a concentration-dependant manner was noticed.

C. cibarius extracts determined the erythrocyte membrane stabilization effect. The erythrocyte membrane is analogous to the lysosomal membrane, and its stabilization implies that the extract may well stabilize lysosomal membranes. Stabilization of the lysosomal membrane is important in limiting the inflammatory response by preventing the release of lysosomal constituents which cause inflammation and damage [44]. All extracts inhibit heat-induced hemolysis at different concentrations (2 – 10 mg/mL extract) (fig. 1). The ethanolic extract displayed the highest degree of protection against heat-induced hemolysis (83.6% ± 1.43% at 10 mg/mL). It is ≈15% higher, as compared with the methanolic extract, and with more or less the same percentage lower than the diclofenac sodium (as a standard).

All extracts presented significant antiproteinase activity at concentrations between 2 and 10 mg/mL (fig. 1). The ethanol extract showed the highest proteinase inhibitory activity, as compared with other extracts. The maximum inhibition decreased in following order: 96.07% ± 0.53% for methanol extract, 95.68% ± 0.6% for cold water extract, and 94.5% ± 1.01% for hot water extract. The diclofenac sodium showed a maximum proteinase inhibitor activity of 99.8% ± 0.06%.

The correlation coefficients between the results obtained for inhibition of albumin denaturation, the heat-induced hemolysis, and proteinase action were determined for all extracts. A statistically significant relationship existed between these methods. For ethanol extract, valoarea R^2 was 0.8192 (inhibition of albumin denaturation vs. heat-induced hemolysis), and 0.9768 (inhibition of albumin denaturation vs. proteinase action), and 0.7801 (inhibition the heat-induced hemolysis vs. proteinase action). The three extracts had a significant degree of correlation. The R^2 value ranged between 0.8136 and 0.8952 (inhibition of albumin denaturation vs. heat induced hemolysis), 0.6572 and 0.9687 (inhibition of albumin denaturation vs. proteinase action), and 0.9241 and 0.9625 (inhibition of heat-induced hemolysis vs. proteinase action). These results could be explained by the difference in bioactive compounds of each extract ($p<0.001$). The active compounds differ from one species to another, but also within the same species due to the geographical differences and the harvesting periods.

Antioxidant components

Natural bioactive extracts are used for the prevention and treatment of multiple human disorders. By contrast with most standard medicinal compounds, these extracts from edible or medicinal mushrooms are often marketed and used by individuals in order to prevent, rather than treat diseases [45]. Their biological activity is directly reflected in their composition of biomolecules with antioxidant and anti-inflammatory effects. Phenols and flavonoids are important groups of biologically active compounds in the mushroom [46].

Within the conducted study, it was identified that homogentisic acid represented the major phenolic component, the presence of which was confirmed by numerous studies, but which varies considerably according to species, and also depending on the geographical harvesting region. It is responsible for protection upon cholesterol degradation [46], but it is also deemed to be involved in the etiology of certain inflammatory processes. In the extracts obtained, the volume of this phenolic acid varied between 1.07 ± 0.03 mg/g and 4.4 ± 0.01 mg/g

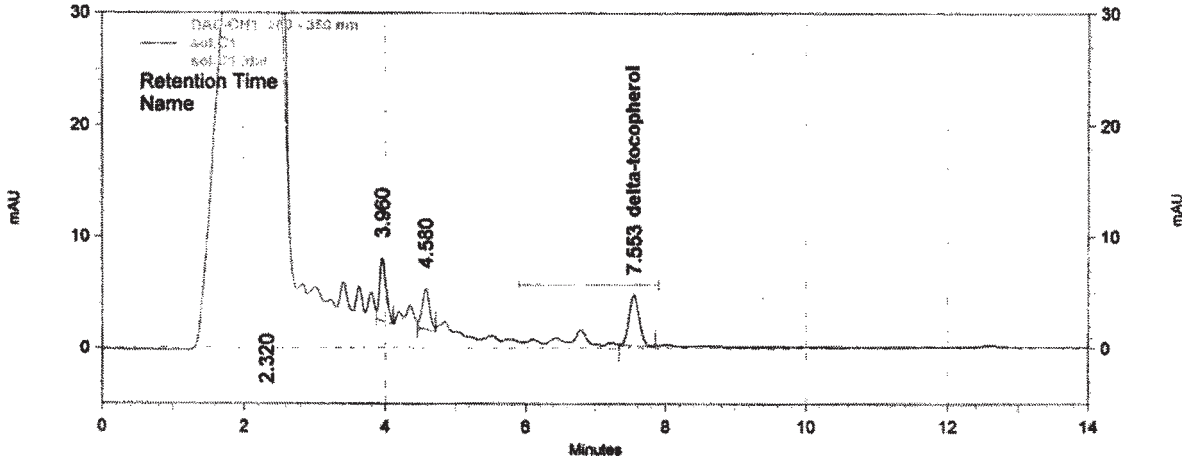

Fig. 3 Chromatogram of δ-tocopherol – ethanolic extract from Cantharellus cibarius

Compounds	Ethanolic extract	Methanolic extract	Hot water extract	Cold water extract
Total phenols*	12.14 ± 1.32	10.4 ± 0.8	9.4 ± 0.56	8.4 ± 0.61
*Homogentisic acid**	4.4 ± 0.01	1.92 ± 0.01	1.07 ± 0.03	1.53 ± 0.01
Gallic acid*	2.1 ± 0.1	0. 07 ± 0.02	0. 03 ± 0.01	0.05 ± 0.01
Total flavonoids (mg quercetin/g extract)	63 ± 2.1	16.5 ± 3.79	4.5 ± 3.88	3.75 ± 1.91
Catechin*	—	—	2.87 ± 0.2	—
Lycopene**	0. 956 ± 0.003	0.406 ± 0.01	0.522 ± 0.001	0.061 ± 0.002
Ascorbic acid*	0.359 ± 0.02	0.24 ± 0.04	0.27 ± 0.03	0.29 ± 0.02
δ-tocoferol*	0.138 ± 0.01	—	—	—

*- mg/g extract

**- μg/g extract

Table 1
BIOACTIVE COMPOUNDS OF DIFFERENT SOLVENT EXTRACTS FROM *CANTHARELLUS CIBARIUS*. EACH VALUE IS EXPRESSED AS MEAN ± STANDARD DEVIATION (n = 3). THE RESULTS WERE SIGNIFICANTLY DIFFERENT $P < 0.05$

extract, and was ≈ 4 higher (the ethanolic extract (table 1), as compared with other researches, but comparable with that from *Colocybe gambosa* [47].

Another phenolic acid identified in extracts was the gallic acid that, in the ethanolic extract, was found in quantities up to 10 times higher (2.1 ± 0.1 mg/g extract), as compared with the same mushroom harvested from Southern Europe. Thus, it is demonstrated that the geographical region has a major influence, because the substrate directly influences the quantity and number of compounds with biological effect, as compared with the studied species. The solvent also has an important role in the isolation of biologically active components, and this was demonstrated by the exclusive presence of catechin, among the flavonoids, in hot water extract, 2.87 ± 0.2 mg/g extract.

In the extracts from fruit bodies of *C. cibarius*, we identified significant quantities of lycopene, especially in the alcoholic extracts (0.956 ± 0.003 μg/g ethanolic extract).

In the extracts obtained, a significant quantity of ascorbic acid was also identified, which was between 0.24 ± 0.04 mg/g and 0.359 ± 0.02 mg/g extract, and higher than the one identified by extraction with ethanol using a Soxhlet system from dry fruit bodies of *Pleurotus ostreatus* (25 mg/100 g) [36].

Also, a quantity of 0.138 ± 0.01 mg δ-tocopherol/g ethanolic extract was identified, which was also emphasized in other studies (fig. 3). This compound can also be found in the species *Lepista inversa, Hygrophoropsis aurantiaca*, and *Laccaria laccata* (in a lower percentage), but approximately equal in various other extracts from *C. comatus* [48]. This compound increases the biological value of the mushroom *C. cibarius*, because the ethanolic extracts can be used in the prevention of cardiovascular dysfunctions.

Using *in vitro* and *in vivo* methods, a deep antioxidant effect of alcoholic and hydrous extracts from the wild edible mushroom *C. cibarius* from the Romanian forests was demonstrated, especially in the case of the ethanolic extract. This extract represents a valuable source of the biologically active compounds (homogentisic acid, carotenoids, δ -tocopherol), and may be useful in extracting natural products with which to fight the effects of free radicals manifested by acute inflammatory processes or dysfunctions of the human cardiovascular system. Future studies will be able to monitor the beneficial effects of consuming these products by using the human gastrointestinal transit *in vitro* stimulation systems (http://www.gissystems.ro/).

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