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TOTAL PHENOLIC ANALYSIS, ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF SOME MUSHROOM TINCTURES FROM MEDICINAL AND EDIBLE SPECIES, BY *IN VITRO* AND *IN VIVO* TESTS

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Abstract

Wild mushrooms are commonly used in various pathologies. However, there are few studies concerning species characteristics from different geographical areas. The aim of the study was to determine the antioxidant and antimicrobial effects of 17 triple tinctures prepared from 9 species of mushrooms with medicinal potential, harvested from the region of Moldova, Romania. The study was conducted in parallel with seven commercial species by in vitro studies (DPPH and ABTS scavenging and chelating activities) and in vivo (antioxidant activity by using Kluyveromyces marxianus yeast strain). A direct correlation of in vitro antioxidant activity to that expressed in vivo was determined in the case of a high content of phenolic compounds. Tincture prepared from Hericium coralloides presented the lowest antiradical capacity. A correlation between the different in vitro antioxidant activities was determined for the Tuber indicum and Piptoporus betulinus species. Tinctures which have a high degree of protection against oxidative action of H_2O_2 had a medium value of $EC_{50} < 1$ mg/mL. The results obtained proved that certain compounds identified in trace amounts may significantly increase the biological effect, as the protocol for tinctures obtaining directly influenced the response and content expressed in vitro and in vivo.

Key words*: extract, dried fruit bodies, scavenging activity, Candida.*

INTRODUCTION

Tincture is a water-alcohol solution rich in biologically active substances of plant origin, obtained from one or more species with medicinal properties. The biological activity *in vitro* and / or *in vivo* is due to the presence of the complex of biologically active substances. The main active molecules are phenolic compounds, which are expressing the therapeutic activity of the final product (tincture) (Gird et al., 2005). From the classical to the modern methods of extraction (ultrasound assisted extraction) all aim to increase the efficiency of active principles extraction, which depends on the species of medicinal herbs or mushrooms used as raw materials (Valachovic et al., 2001).

In the case of fungi, tinctures utilization is less widespread. In Romania, administration of extracts from medicinal mushrooms is made in dry form, as lyophilized or atomized powder. Traditional medicinal species are used: *Lentinula edodes, Ganoderma lucidum, Agaricus brasiliensis, Hericium erinaceus*. Using these species and many others as well, that is known by traditional medicine, has been driven by the need to find new biologically active compounds from natural sources, which will not cause side effects (Lindequist et al., 2005). The most frequent use of these extracts is the antitumor one, as a supplement to the chemotherapeutic medication (Safarzadeh et al., 2014). In this study, it was determined the effect of antioxidant and antimicrobial triple tinctures obtained from 13 species of mushrooms with medicinal potential, harvested in Moldova region, Romania, plus seven commercial species and a control of plant origin (*Juniperus communis* L.). All results have been correlated with the amount of phenolic compounds ascertained in each tincture.

MATERIALS AND METHODS

Samples. Nine of wild medicinal species (*Ganoderma applanatum, Trametes hirsuta, Perenniporia fraxinea, Fomitopsis pinicola, Ganoderma australe, Trametes ghibosa,*

Lenzites betulina, Piptoporis betulinus, Schizophillum commune) was harvested from Moldova region, Romania in automn of 2013 and spring 2014. *Craterellus tubaeformis, Hypomyces lactifluorum, Volvariella volvacea* was buy from Oregon Mushroom, USA. *Tuber indicum* was by from Metro Graz, Austria. Also, three dried edible species was used (*Auricularia judae, Boletus edulis* and *Cantharellus cibarius*) bought from supermarket. *A Juniperus communis L. (SC Stef Mar SRL Râmnicu Vâlcea, Romania)* bush fruit was used as a control.

Preparation of triple tinctures. Fifty grams of dried mushroom species were extracted with 250 mL ethanol 50% for fifteen days, at room temperatures with a manual daily agitation. The obtained extract was supplemented with the same solvent and mixed with new dried samples (same quantities), repeating the same protocol twice. Each extracts was filtered with Whatman No. 4 filter paper (Essaidi et al., 2013).

Determination of antioxidant activity, *in vitro*. Chelating activity, DPPH and ABTS scavenging activities were determined spectrophotometrically (517 nm, respectively 415 nm), according following formula: scavenging activity (%) = $[(A_C - A_S)/A_C]\times100$, where A_C is the absorbance of the control and A_S is the absorbance of the sample (Liu et al., 2013).

Determination of antioxidant activity, *in vivo*. Antioxidant activity *in vivo* was presented in a previous study with slightly modification. Yeast strain *Kluyveromyces marxianus* was used and the viability was read to Colon Quant, by comparison with a sample of untreated cells (Vamanu & Nita, 2014a).

Determination of antimicrobial capacity. The antimicrobial capacity was tested *in vitro* against certain strains of bacteria and yeasts obtained from the Collection of the Faculty of Biology, University of Bucharest (Table 2, Vamanu, 2017). For this protocol, it was used a Bioscreen C MBR and a serial dilution was made in a proper media. After 48 hours the optical density was read at 600 nm and expressed as the minimum inhibitory concentration (MIC) of tinctures which means the minimum concentration that inhibited the tested strains growth.

RESULTS AND DISCUSSIONS

1. *In vitro* determination of the antioxidant activity.

In order to assess the antioxidant capacity, *in vitro* and *in vivo* tests was conducted, leading to point out the specific properties of each tincture. Even if an thorough evaluation requires the use of several solvents in parallel, in the case of tinctures for human use other alcohols cannot be used to demonstrate the effects of the composition in biologically active substances. The quantification of the antioxidant effect, demonstrated by free radical scavenging and chelating activity, was achieved in all tested tinctures and was presented in **Table 1**. The tincture prepared from *H. coraloides* presented the lowest ($p<0.04$) antiradical capacity (EC_{50} values were 16.07 and 21.77 mg/mL), whereas *P. betulinus* and *T. indicum* displayed the lowest chelating capacity ($p<0.05$). For this parameter, the EC_{50} value of the two species was about the same, and was not correlated with the antiradical one.

Although a study about the activity of such medicinal mushroom extracts is lacking, the results could be appreciated as appropriate to the studies demonstrating that a hydro-alcoholic solvent will result in the presence of a high concentration of antioxidant compounds (Martins et al., 2015; Vamanu, 2013a). Compared with another widely used solvent (methanol), the triple tincture of *P. betulinus* presented a similar EC_{50} value for the scavenging activity against DPPH radicals (Reis et al., 2011). Only in 7 species of fungi were determined EC_{50} values which were lower than 1 mg/mL for DPPH scavenging activity.

Table 1 presents the values for the power reduction expressed by tested tinctures. A correlation was observed with previous values for species *Tuber indicum, Piptoporis betulinus* and *Hericium coraloides* because they had the lowest reducing power and DPPH scavenging capacity. Tinctures that determined a maximum donation capacity of an electron were *Ganoderma australe* and *Craterellus tubaeformis*. The EC_{50} index showed values of up to 1 mg/mL.

2. Determination *in vivo* of the antioxidant activity.

The protective action exerted *in vivo* is performed by inhibiting the reactions resulting in formation of free radicals, by means of phenolic component (in particular). The absorption of these biologically active compounds will cause an increase in the reaction capacity of the organism at the cellular level (Silva et al., 2009). By using the tinctures of mushroom dried fruit bodies an increase was recorded in the resistance of eukaryotic cells, *K. marxianus*, to the stress caused by reactive oxygen species generated by the decomposition of hydrogen peroxide. As shown in **Table 1**, the protective action was correlated with the decreased pro-oxidant action of metals in transition, by their transformation in more stable complexes (Koncic et al., 2011). These results are directly influenced by the membrane absorption of some flavonoids (e.g., catechin), to which may be added the total amount of sugars present in the samples, which could directly influence cell viability (Stefenon et al., 2010).

Thus, was ascertained the ability of tinctures to protect the cells that are in the exponential growth phase to oxidative stress induced in the presence of H_2O_2. As expected, the cells of *K. marxianus* displayed different degrees of sensitivity to the oxidative treatment. Depending on the exerted effect **(Table 1),** only the tinctures T4 - T10 showed a high degree of protection against the oxidative action of H_2O_2, with an average EC_{50} value of <1 mg/mL. Instead, tinctures T11 - T17 had a reduced protection level to the oxidative stress. In this case, cell viability presented low values, of below 30%. The first three tinctures had a mean degree of protection, of around 46 ± 3.47%. T4 - T6 determined an increase of the average viability of about 25-30%.

Compared to previous *in vivo* studies, increasing cell viability of *K. marxianus* was correlated with an increase in glutathione peroxidase and reductase enzyme activity, if the studies are related to an untreated control. The protective effects are correlated to the polyphenolic content. Thus, certain phenolic compounds had a stimulating effect on the metabolism of yeast cells, which would be translated in increasing the viability after the oxidative stress. Among these compounds, the stimulating cause of this behaviour would be anthocyanins, compounds found in plants and in fungi as well, having a direct role in the vascular protection and in stimulation of the cognitive processes (Baroni et al., 2012; Cho et al., 2003).

In any event, the antioxidant effects *in vitro* and *in vivo* are never the result of only one single class of bioactive compounds. They are the result of complex interaction between several classes of compounds that exert their pharmacological effects in certain situations. As regards the *in vivo* effect, the digestive activity should not be ignored, as it has a direct influence on the activation of the phenolic component. Facilitating the absorption of valuable components will be the result of a correct functioning of various physiological functions that are related to the structure and fermentation activity of human colonic microbiota.

3. Determination of antimicrobial capacity.

Evaluation of antimicrobial activity expressed as MIC demonstrated an inhibitory capacity of all tinctures. **Table 2** shows that from all edible species only *Auricularia judae* and *Cantharellus cibarius* had a significant inhibitory activity against *Candida* strains ($p<0.05$). Otherwise, from the wild species of mushrooms, only with the tincture of *Phellinus pomaceus* were obtained low MIC values, of about 8 mg/mL. For most species modest MIC values were obtained, requiring high concentrations, of at least 16 mg/mL.

The inhibitory activity against Gram - positive strains was especially expressed in the case of *Staphylococcus aureus*, which is known for its resistance and high infectivity. Species *Trametes versicolor*, *Trametes hirsuta* and *Perenniporia fraxinea* showed inhibitory activity against *B. cereus* and *L. innocua*, widespread in nature, causing food poisoning. The antimicrobial activity against these strains is determined, according to previous studies (Vamanu, 2017), by the flavonoid component. The researches have shown the effect caused by the presence of myricetin and the mentioned tinctures may contain the compound that has also been identified in extracts from the fungus mycelium (Vamanu, 2014). In addition, Gram-

negative strains are known to present a significant resistance to the alcoholic or aqueous plant extracts (Oh et al., 2013). This aspect was also described in the case of these tinctures obtained from edible and/or medicinal mushrooms, for example against *E. coli*. A larger number of tinctures have been identified, having a MIC value of 64 µL/mL.

To all this is added the presence of polysaccharides, which can in turn bind phenolic compounds, thus participating in the expression of the biological value. Even the alcohol will favour the presence of such polysaccharides, which often can be seen on the bottom of the brown bottles in which the tinctures are stored. Tinctures of mushrooms (eg *Hericium coraloides*, *Trametes versicolor*, *Piptoporis betulinus*) display, after stored overnight in a refrigerator, a white deposit of polysaccharides. At the moment when the solutions reach room temperature, the polysaccharides are re-dissolved.

Table 1. Antioxidant activity (EC_{50} values, mg/mL) of mushroom tinctures

Tinctures (mushroom species)	DPPH scavenging activity	ABTS scavenging activity	Chelating activity	*In vivo* antioxidant activity
T1 (*Auricularia judae*)	5.04	0.56	5.67	3.23
T2 (*Cantharellus cibarius*)	8.97	7.67	4.07	4.62
T3 (*Boletus edulis*)	4.67	8	2.87	6.69
T4 (*Tuber indicum*)	1.2	7	12.56	0.57
T5 (*Craterellus tubaeformis*)	0.6	0.27	1.33	0.50
T6 (*Hericium coraloides*)	16.07	21.77	5.98	0.55
T7 (*Fonies fomentaris*)	0.6	0.14	5.80	0.70
T8 (*Trametes versicolor*)	1.2	0.2	2.54	0.73
T9 (*Phellinus pomaceus*)	0.7	0.1	3.61	0.63
T10 (*Trametes hirsuta*)	1.3	4.34	6.87	0.97
T11 (*Perenniporia fraxinea*)	1.5	0.04	4.63	> 50
T12 (*Fomitopsis pinicola*)	0.7	0.24	1.03	> 50
T13 (*Ganoderma australe*)	0.5	0.1	0.71	> 50
T14 (*Trametes ghibosa*)	0.9	0.14	1.48	> 50
T15 (*Lenzites betulina*)	1.2	1.07	4.76	> 50
T16 (*Piptoporis betulinus*)	4.57	7.02	13.76	> 50
T17 (*Schizophillum commune*)	0.5	0.3	3.02	> 50

Table 2. Antimicrobial activity (µL/mL) of mushroom tinctures

Tinctures (mushroom species)	*Listeria innocua*	*Bacillus cereus*	*Escherichia coli*	*Pseudomonas aeruginosa*	*Staphylococcus aureus*	*Candida sp.*	*Candida albicans*
T1 (*Auricularia judae*)	32	16	64	16	32	32	8
T2 (*Cantharellus cibarius*)	32	16	32	16	16	16	8
T3 (*Boletus edulis*)	64	64	64	32	32	8	8
T4 (*Tuber indicum*)	64	64	32	16	32	32	32
T5 (*Craterellus tubaeformis*)	16	64	64	32	32	32	32
T6 (*Hericium coraloides*)	16	8	16	16	8	32	16
T7 (*Fonies fomentaris*)	32	32	32	32	8	32	32
T8 (*Trametes versicolor*)	8	8	16	32	8	32	32
T9 (*Phellinus pomaceus*)	32	16	16	32	8	16	8
T10 (*Trametes hirsuta*)	8	8	8	32	8	16	16
T11 (*Perenniporia fraxinea*)	8	8	8	16	8	32	32
T12 (*Fomitopsis pinicola*)	16	32	8	16	16	32	16
T13 (*Ganoderma australe*)	16	8	16	32	16	32	16
T14 (*Trametes ghibosa*)	8	32	8	8	16	16	16
T15 (*Lenzites betulina*)	32	16	16	32	8	32	32
T16 (*Piptoporis betulinus*)	32	32	16	16	8	16	32
T17 (*Schizophillum commune*)	16	16	16	16	8	32	16

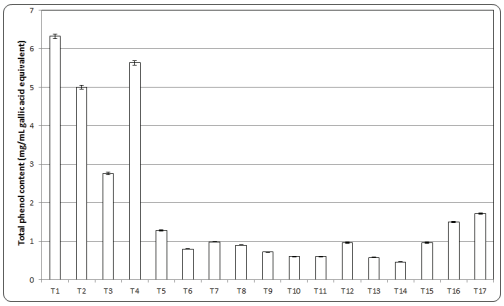

Figure 1. The total phenolic content in mushroom tinctures

4. Determination of total phenolic and flavonoidic compounds

The total composition in phenolic compounds is shown in **Figure 1**. The highest content in phenolic compounds was determined in the tinctures of *Tuber indicum*, *Cantharellus cibarius* and *Auricularia judae*, with a content exceeding 5 mg/mL gallic acid equivalent. Instead, the tinctures T6 - T15 had the lowest content of maximum 1 mg/mL gallic acid equivalent. These results demonstrate that the assessed biological effects, *in vitro* and *in vivo*, were primarily due to the content in flavonoids, whose maximum values were correlated with the tinctures whose biological activity was maximal. These findings do not mean that the phenolic compounds, that most frequently are represented by gallic acid (Vamanu & Nita, 2014a), protocatechuic acid or phydroxybenzoic acid (Leal et al., 2013) do not participate in the expression of antioxidant effect, for example, but have only a secondary role (**Figure 2**).

In this situation, a direct correlation was revealed of these compounds with the antioxidant activity *in vitro*. Tinctures derived from species *Craterellus tubaeformis* and *Piptoporis betulinus*, had values that exceeded 100 µg / mL catechin equivalent. An exception was obtained with the tincture of the species *Perenniporia fraxinea*, which exceeded by approximately 10% the previously mentioned value, but this was only slightly correlated with the antioxidant activities *in vitro* and *in vivo*. It is better correlated with the antimicrobial activity, which shows that in the same species there is not a direct correlation between the antioxidant and the antimicrobial activity. These studies disagree with other previous results corresponding to the mycelia of the species *Pleurotus ostreatus* and/or *Coprinus comatus* (Vamanu, 2013a).

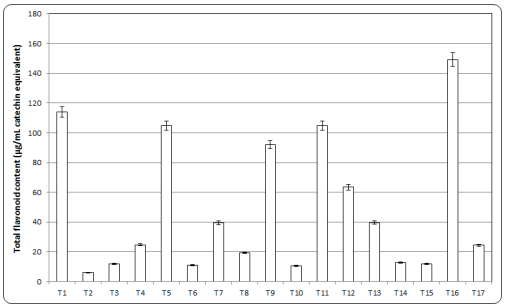

Figure 2. The total flavonoid content in mushroom tinctures

Due to the various biological processes by which acts, a direct correlation between the biological activities has not been found. The highest R^2 values were determined for *Craterellus tubaeformis*; for example a value of approximately 0.745 was obtained between the scavenging activities and the reduction power of the Fe ion. This value was as well appropriate in the case of a correlation with *in vivo* antioxidant activity.

The chelating activities the values decreased by about 30%. Instead, the antimicrobial activity was primarily correlated to the total phenolic content. According to some previous studies it depends on the combination of various phenolic compounds and on the chemical structure (Vamanu, 2013b; Sandigawad & Patil, 2010). If we refer to recently published studies, a significant part of the antioxidant answer, expressed at least *in vitro*, is due to the polysaccharide component, which would correspond to the non-correlation between the decreased phenolic content and the EC_{50} value expressed by some species (eg, *Hericium coraloides*). Thus, a direct correlation of the *in vitro* antioxidant activity to that expressed *in vivo* was determined in the case of a high content in phenolic compounds. These results were consistent to a content that exceeded 6 mg/g gallic acid equivalents for *Agaricus bisporus* (Liu et al., 2013). Also, the research results have shown the presence among the phenolic compounds not only of caffeic acid and homogentisic acid but also of the

flavonoids miricitine and routine (Liu et al., 2013; Vamanu & Nita, 2014b).

CONCLUSIONS

The results do not directly certify the relationships between the content of biologically active compounds and the biological activities *in vitro* and/or *in vivo*, but indicate that certain compounds identified in trace amounts may significantly increase the expressed effect. The same situation was registered in the case of identification of some specific phenolic compounds in medicinal plants (Vamanu & Nita, 2013). Thus, the various technological processes of extraction directly influenced the content and the biological response expressed *in vitro* and *in vivo*. Optimization of obtaining the tinctures from the fructification body of edible and medicinal mushrooms is underway, aiming at increasing the content of these bioactive compounds, and also at finding other possible effects. Tests are under consideration regarding the effect of absorption and digestion processes on the stability of the chemical composition.

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