ORIGINAL ARTICLE

IN VIVO ANTIOXIDANT PROPERTIES OF SOME MUSHROOM EXTRACTS IN EXPERIMENTALLY INDUCED DIABETES

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Abstract

Mushrooms contain compounds with different biological properties such as vitamins, minerals, polyphenols and flavonoids. The aim of our study was to evaluate the capacity of some extracts from mushrooms to restore endogen antioxidant systems in experimentally induced diabetes in mice. Methanolic extracts from the following mushrooms have been used: *Agaricus bisporus* brown, *Pleurotus ostreatus* and *Fomes fomentarius*. All extracts increased the value of superoxide dismutase (SOD) from 96.40 ± 1.35 U/mL blood in diabetes group to 260.60 ± 9.97 U/mL blood in *F. fomentarius* group. The value of catalase decreased in diabetes group (6.57 ± 0.20 U/mg proteins) compared to the control group (10.34 ± 0.34 U/mg proteins). *F. fomentarius* extract had the capacity to restore catalase activity to 10.26 ± 0.25 U/mg proteins compared to the control group. The value of thiobarbituric acid reactive substances (TBARS) decreased from 1.78 ± 0.03 nmols/mg proteins in diabetes group to 1.29 ± 0.01 in *A. bisporus* brown group, and respectively to 1.25 ± 0.07 in *F. fomentarius* group.

Rezumat

Ciupercile conţin diferiţi compuşi cu acţiune biologică cum sunt vitamine, minerale, polifenoli şi flavonoide. Studiul a urmărit evaluarea capacităţii unor extracte din ciuperci de a reface sistemele antioxidante endogene in diabetul indus experimental la şoareci. Au fost analizate extracte metanolice din următoarele ciuperci: *Agaricus bisporus brown*, *Pleurotus ostreatus* şi *Fomes fomentarius*. Extractele au determinat creşterea valorii superoxid dismutazei (SOD) de la 96,40 ± 1,35 U/mL sânge în grupul diabetic la 260,60 ± 9,97 U/mL în grupul tratat cu extract din *F. fomentarius*. Valoarea activităţii catalazei a scăzut în grupul diabetic (6,57 ± 0,20 U/mg proteine) comparativ cu grupul control (10,34 ± 0,34 U/mg proteine). Extractul din *F. fomentarius* a restabilit activitatea catalazei la 10,26 ± 0,25 U/mg proteine, valoare apropiată de a grupului control. Extractele au redus valorile *thiobarbituric acid reactive substances* (TBARS) de la 1,78 ± 0,03 nmoli/mg proteine în grupul diabetic la 1,29 ± 0,01 în grupul tratat cu extract din *A. bisporus* brown şi la 1,25 ± 0,07 în grupul tratat cu extract din *F. fomentarius*.

Keywords: mushrooms, *Agaricus bisporus* brown, *Pleurotus ostreatus*, *Fomes fomentarius,* catalase, SOD, TBRAS

Introduction

The incidence of diabetes increased considerably in the last years and became the third serious chronic disease following cancer and cardiovascular diseases. The prevalence of diabetes is increasing, and the number of cases is predicted to reach approximately 592 million by 2035 [4].

Chronic hyperglycaemia increases the intracellular concentration of reactive oxygen species (ROS), disturbs mitochondria functions, impairs insulin secretion and induces inflammation [12]. On the other hand, chronic hyperglycaemia will cause changes in the structure and functions of erythrocytes membrane by increasing their aggregation ability and reducing the deforming capacity. Oxidative stress is involved in the development and progression of diabetes complications such as retinopathy, nephropathy and neuropathy, impairing of cardiac morphology and functionality, and finally accelerates the aging of the organism [19, 23].

The use of double-acting substances capable to control postprandial hyperglycaemia and to reduce oxidative stress by directly blocking oxidants or by stimulating endogenous antioxidant defence mechanisms can be extremely useful for patients with diabetes or pre-diabetes.

Polysaccharides and their protein complexes, dietary fibers, and other components extracted from fruiting bodies or cultured mycelia have anti-hyperglycaemic properties [11, 13, 25]. The mycelium of mushrooms enriched with selenium contains

more selenoproteins that will improve antidiabetic properties [10].

Polyphenols possess a high reactivity, as hydrogen or electron donors have the ability to stabilize radicals and to chelate transition ion metals that induce ROS synthesis [16]. Mushrooms and their extracts contain polyphenols and flavonoids that have the ability to block reactive oxygen species, and have anti-inflammatory, immunomodulatory and anticancer effects [7].

In order to evaluate the *in vitro* and *in vivo* anti-oxidant effects of mushrooms, we used extracts, respectively powder obtained from two edible mushrooms (*Agaricus bisporus* brown, *Pleurotus ostreatus*) and one non-edible mushroom *Fomes fomentarius*.

A. bisporus brown contains high quantities of dietary fiber, antioxidants, linoleic acid, vitamin C, D2 and B12, folate and polyphenols. *A. bisporus* is the most used mushroom in human nutrition from *Agaricus* species. Studies on rats have shown that mushroom powder can reduce cholesterol and triglyceride levels, especially if is associated with other high-fiber vegetable powders [7].

P. ostreatus is an edible mushroom widely used in human nutrition. It contains polyphenols, β-glucan, with antioxidant, immunomodulatory, antidiabetic, lipid lowering and antitumor effects [26].

F. fomentarius is used in oriental medicine for its antioxidant, anti-inflammatory, antitumor and antibacterial properties [21]. These effects are induced by polyphenols, flavonoids, sterol derivatives and glucans [8, 22].

Materials and Methods

Vegetal products

A. bisporus brown (Abb) - 3 samples, *P. ostreatus* (Po) - 2 samples (Po1 was cultivated; Po2 was collected from the forest), *F. fomentarius* (FF) - 1 sample.

5 g dried sample was extracted with 100 mL methanol at 80^{o}C. The methanol extract was evaporated to dryness under reduced pressure at 40^{o}C.

In vitro antioxidant properties of extracts were evaluated by inhibition of hemolysis mediated by peroxyl free radicals: 0.1 mL 10% suspension of erythrocytes in 10 mM phosphate-buffered saline (PBS) at pH 7.4 was added to 0.2 mL of 200 mM 2,2'-azobis(2-amidinopropane) dihydrochloride (AAPH) solution and 0.1 mL of mushroom extract of different concentrations (solved in dimethyl sulfoxide). After incubation for 3 hours at 37^{o}C the reaction mixture was diluted with 4 mL PBS and centrifuged at 3000g for 10 min; the absorbance of the supernatant was read at 540 nm [14]. The rate of haemolysis inhibition was calculated by the equation: %haemolysis inhibition = $[(A_{AAPH} - A_S)/A_{AAPH}] \times 100$, where A_S is the absorbance of the solution containing the mushroom extract and A_{AAPH} is the absorbance of the solution without the mushroom extract. The extract concentration providing 50% inhibition (EC_{50}) was calculated and expressed as μg extract/mL final solution. L-ascorbic acid was used as positive control. All samples have been analysed in triplicate. For each sample, the mean and the standard deviation has been calculated.

In vivo antioxidant evaluation

Animals - mice with streptozotocin-induced diabetes (50 mg/kg, intraperitoneal). The experimental procedures were conducted with the approval of the Ethics Committee of the "Grigore T. Popa" University of Medicine and Pharmacy Iaşi, Romania and in accordance to the European Communities Council Directive 86/609/EEC.

The extract, respectively the mushroom powder were administered to mice (200 mg/kg body weight, orally for 2 weeks). Animals received standard food and water *ad libitum*.

The experimental groups (10 animals) were: C - control (citrate solution); D - diabetes; AbbE - diabetes + Abb extract; AbbP - diabetes + Abb powder; PoE - diabetes + Po extract; PoP - diabetes + Po powder; FF - diabetes + Ff extract. After 2 weeks, the animals were sacrificed and the blood was collected on vacutainer with anticoagulant. The values of catalase (CAT) and superoxide dismutase (SOD) activity in haemolysed blood, respectively thiobarbituric acid reactive substances (TBARS) in plasma, were determined.

SOD determination (RANSOD kit): xanthine oxidase catalyses the oxidation of xanthine and generates superoxide radicals which react with 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyltetrazolium chloride to form a red formazan dye. The superoxide dismutase reacts with the superoxide and reduces the absorbance of solution at 505 nm [2].

Catalase determination: catalyse from haemolysed blood reacts with hydrogen peroxide in phosphate buffer and decrease the absorbance at 240 nm. The time necessary to reduce the absorbance from 0.450 to 0.400 is recorded and used to calculate the enzyme activity [1].

TBARS determination: plasma is mixed with 1 mM ethylenediaminetetraacetic acid solution, 10% tri-chloroacetic acid; after centrifugation, the super-natant is mixed with 0.5% 2-thiobarbituric acid and heated to 100ºC for 15 minutes. Solution is cooled and the absorbance is read at 535 nm [20].

All samples have been analysed in triplicate. For each sample the mean and the standard deviation have been calculated.

Results and Discussion

Our previous studies indicate that *F. fomentarius* extract contained the highest amount of polyphenols (8.58 ± 0.08 mg gallic acid equivalent/g dried extract) and *P. ostreatus* (Po2) contained the most important quantity of flavonoids (2.62 ± 0.05 mg catechin equivalent/g dried extract). For Abb and Po samples the quantity of polyphenols is higher when the cap of mushrooms is dark coloured and present small-medium size [16].

In vitro antioxidant properties

The extracts protect the membrane of erythrocytes against oxidation induced by AAPH in a dose dependent manner (Table I).

Table I

The values of EC_{50} (μg/mL) for the inhibition of haemolysis of the mushroom methanolic extract

Sample	Concentration (mg/mL)/haemolysis inhibition (%)				EC_{50} (μg/mL)
	2.5	5	10	20	
Abb1	20.19 ± 0.12	35.08 ± 0.32	56.12 ± 0.19	68.43 ± 0.52	173.90 ± 1.30
Abb2	21.04 ± 0.11	39.34 ± 0.51	64.74 ± 0.84	77.19 ± 0.87	142.33 ± 2.52
Abb3	25.92 ± 0.42	41.17 ± 0.33	66.50 ± 0.49	80.49 ± 0.90	135.46 ± 1.43
Po1	12.42 ± 0.17	27.08 ± 0.20	50.13 ± 0.23	60.22 ± 0.74	211.93 ± 1.46
Po2	20.12 ± 0.30	40.49 ± 0.17	66.92 ± 0.37	79.24 ± 0.67	136.52 ± 0.86
FF	30.24 ± 0.39	50.33 ± 0.72	71.04 ± 0.41	86.07 ± 0.85	105.23 ± 2.59
Ascorbic acid	62.09 ± 0.95	74.56 ± 1.09	88.23 ± 1.24	97.08 ± 1.17	54.17 ± 1.07

The protective effects of the extracts depend on their composition. FF extract contained the highest amount of polyphenols and presented the most intensive protective effect but twice less than ascorbic acid effect. Po2 contained a lesser amount of polyphenols than Abb3, but the value for EC_{50} was similar. This could be explained by the presence of flavonoids in Po2 (2.62 ± 0.05 mg/g) more than Abb3 (1.52 ± 0.01 mg/g). Nowacka *et al* described for *F. fomentarius* the most important antioxidant properties compared with other wild growing mushrooms [17].

The EC_{50} values were lower than those determined by Barros *et al* that analysed mushrooms collected from the forest. Mushrooms from natural environmental produce more polyphenols and flavonoids for their protection and the extracts obtained from these have more important antioxidant effects [3].

In vivo antioxidant tests

For the *in vivo* evaluation, we selected the most active samples on the *in vitro* tests: FF, Po2, and Abb3.

The food intake increased up to 12.40% and water consumption increased up to 9.97% in diabetes group comparing to the control group. By treating mice with the extract and the powder of mushrooms, the food and water consumption decreased compared to the diabetes group, especially after PoP2 administration (27%, respectively 34.48%). The FF extract decreased the glucose level by 31.35% compared to diabetes group [16].

Mushrooms extracts induced a significant reduction of postprandial serum glucose by different mechanisms and can reduce oxidative stress that is a consequence of hyperglycaemia [6].

SOD protects red cells against oxidants and extracellular SOD is important to ensure normal vascular function and cardiovascular health. Determination of superoxide dismutase (SOD) revealed an improvement in enzyme activity ($p < 0.0001$) in groups treated with extract or powder, reaching values higher than the control group (Figure 1).

Catalase decomposes hydrogen peroxide and protects lipids and proteins against oxidation. The value of catalase decreased in diabetes group by comparing with control group ($p < 0.0001$) (Figure 2). FF extract had the most important capacity to restore catalase activity compared to the control group ($p = 0.5563$).

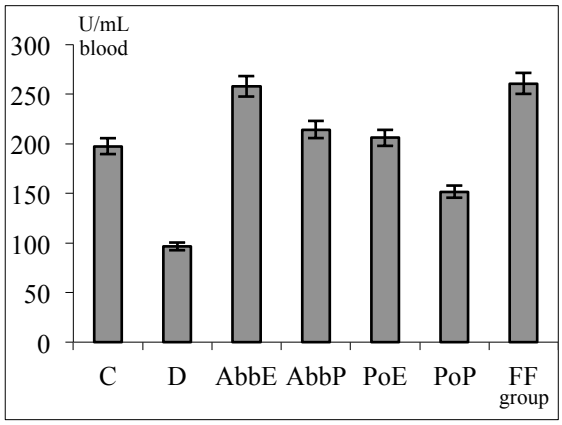

Figure 1.
The values obtained for SOD (U/mL blood) on experimental groups

Administration of extracts and powder restored the activity of SOD and CAT depending on the polyphenolic concentration. FF that contains the highest amount of polyphenols had the best effect. On both tests the powder was less active than the extract.

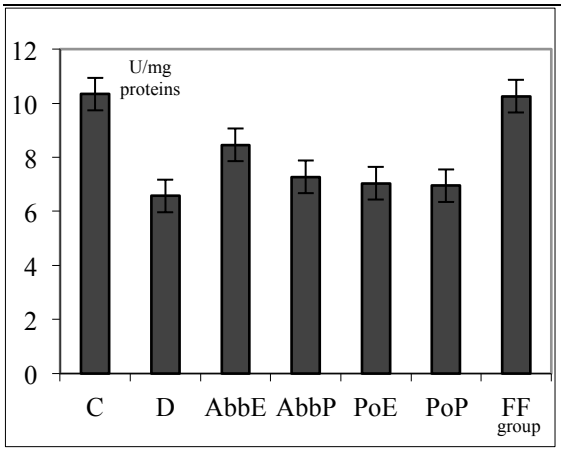

Figure 2.
The values obtained for catalase (U/mg proteins) on experimental groups

P. ostreatus extract increases catalase gene expression in aged rats and increases the efficacy of endogenous antioxidant systems. Also, the extract decreases the rate of protein oxidation by free radicals [5].
TBARS represent a group of compounds formed by oxidation of lipids and proteins. These compounds are markers for oxidative stress. Malondialdehyde (MDA) is the end product of lipid peroxidation and is the most important compound from TBARS group (Figure 3).

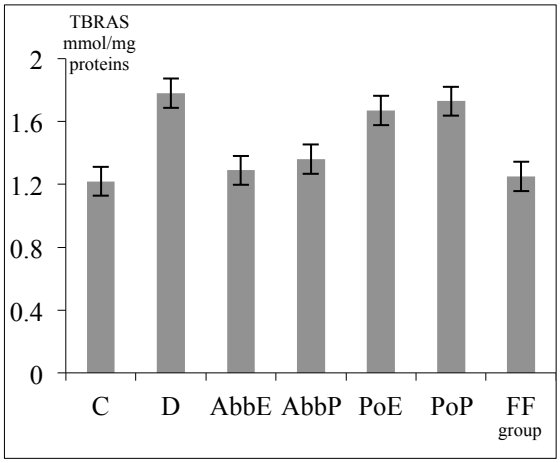

Figure 3.
The values obtained for TBRAS (mmol/mg proteins) on experimental groups

AbbE, AbbP and FF decreased the amount of TBRAS ($p < 0.0001$), and the effect of PoE and PoP were not so important ($p = 0.3856$, respectively 0.6046).
The more intensive activity of extracts could be explained by the highest bioavailability of antioxidant compounds from the extract than from the powder. Fibers presented in great quantity in powders will decrease the rate of absorption for the active compounds.

The study of Likidlilid *et al* indicated that MDA level in red cells from diabetic patients was higher than for normal subjects and the highest values were determined to patients with diabetes and coronary heart disease. A significant correlation has been found between the level of MDA, CAT activity and fasting plasma glucose [9]. We observed the same effect for our extracts, thus Abb samples and FF decreased glucose levels and improved oxidative stress biomarkers more than Po samples.
The level of MDA increases in uncomplicated or complicated diabetes. MDA for diabetic patients showed a significant positive correlation with plasma glucose, lipid profile parameters, and significant negative correlation with Zn and Mg. These data indicated the connection between hyperglycaemia and oxidative stress [16].
Mycelium powders of *Agaricus brasiliensis* and *Ganoderma lucidum* have membrane protective properties for red blood cells, normalize their aggregation properties, and suppress the apoptosis of leukocytes [24].
Polysaccharides extracted from *P. ostreatus* reduce the risk of oxidative damage by increasing SOD, CAT, and glutathione peroxidase activities and decreasing MDA level [26].

Conclusions

Our study showed that diabetes decreases the potency of endogen anti-oxidant systems by decreasing SOD and CAT enzymes activities and increasing MDA synthesis.
Oxidative stress in diabetes will modify the ability of red blood cells to transport oxygen, the rheological properties of blood and the functions of immunocompetent cells. Also, the oxidative stress is involved in the development of micro- and macro-angiopathies that are the complications of diabetes.
The mushrooms' extracts studied presented more important antioxidant properties than the powder probably due to active compounds that are easily released and absorbed out of these, compared to powder. Antioxidant properties depend especially on the polyphenols content.

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