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Research article

Chemical Study of Some Fungi: Evaluation of their Antioxidant and Xanthine Oxidase Effects

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Abstract

The chemical composition of aqueous extracts from four fungal fruiting bodies, called Amanita muscaria, Amanita Caesarea, Agaricus campestris and Agaricus silvicola was analyzed. In these aqueous extracts, 4 different chemical families were measured as polyphenols, tannins, flavonoids and antocyanins. This study also focuses on the study of the antioxidant activities ABTS / DPPH. Agaricus campestris and Agaricus silvicola have the most important antioxidant activity with the DPPH with an IC50 of 65.3 ± 1.59 mg / ml and 80.9 ± 1.7 mg / mL, respectively. Agaricus campestris and Amanita muscaria have the most important antioxidant activity with ABTS with an IC50 of 45.6 ± 0.8 mg / ml and 79.69 ± 1.9 mg / mL, respectively. The observation of flavonoids/polyphenols concentration ratio seems to explain this results disparity in antioxydants. Finally, we determined the inhibitory xanthine oxidase activity of four aqueous extracts against, whose three fungi are traditionally used in French cuisine. The level of XOD inhibitory activity was higher in the aqueous extract of Agaricus campestris (IC50 32.55 ± 1.09 mg / ml), followed by that of Amanita Ceasarea (IC50 of 45.1 ± 1.2 mg / ml) and finally that of Agaricus silvicola (IC50 of 61.55 ± 3.1, very good 3 edible.

Keywords: fungi, chemical composition, antioxidant activity, XOD inhibitory effect

Introduction

Natural products with antioxidant system can help our endogenous defense. Along this axis, the antioxidants in the diet are of major importance as agents of protection that reduce oxidative damage. Although plants are widely studied in this area, the antioxidant properties of wild mushrooms also draw attention of today's research, and many antioxidant compounds extracted from these sources have been identified, such as phenolic compounds, tocopherols, ascorbic acid, and carotenoids.

Amanita muscaria (L.) Lam, belonging to the family Amanitaceae, is toxic ectomycorrhizal fungus with a wide host range [1]. Widespread, Amanita muscaria is a native of temperate and boreal forest regions of the Northern Hemisphere. Popular for its hallucinogenic properties, Lewis Carroll was inspired by Amanita muscaria to write his classic tale, Alice in Wonderland [2]. Intoxication with A. muscaria is characterized by the following symptoms: dizziness, nervousness, euphoria, excitement, drowsiness, altered perceptions, nausea, vomiting and diarrhea, muscle spasms, numbness in the limbs [3]. Its two main active constituents are muscimol and ibotenic acid, which are derivatives of isoxazole responsible for its hallucinogenic properties [4,5]. Amanita muscaria, although having hallucinogenic properties, remains an attractive source of bioactive molecules, in fact, we know that alpha amanitin and phalloidin, the two toxic compounds have antioxidant properties [6,7].

The identification of A. muscaria is relatively easy, though, when there is heavy rain, its fruiting body may lose its distinctive white patches, then A. muscaria may look like A. ceasarea Amanita ceasarea (Scop.) Pers. (Caesar's Mushroom), belonging to the same family, native to southern Europe and northern Africa, is rather a very good edible wild mushrooms considered, making the consequences of the confusion, severe. Malic and ascorbic acid, most abundant compounds of Amanita Caesarea, justify the nutritional value of this fungus [8]. Agaricus campestris (L., 1753) (field mushroom) was found to be good sources of protein and total carbohydrate, with 27.95 and 42.62 g/100 g, respectively. Moreover, it has a very low fat content of 1.34 g/100 g [9]. Agaricus silvicola (Vittad.) Peck (1872) (wood mushroom) contains 238.7 mg / kg of p-hydroxybenzoic [10].

The mushrooms are widely appreciated worldwide for their

potential nutritional and pharmacological value as sources of important bioactive compounds [11,12]. That is why we seek to complement the knowledge of 4 of them. The aim of our work on fungi was to study the chemical composition of their aqueous extracts in the families of chemicals such as polyphenols, tannins, flavonoids and anthocyanins. These extracts were also evaluated for their antioxidant activity

Finally, the enzyme activity against Xanthine Oxidase (XOD) was also studied for these samples. Xanthine oxidase (XOD), is considered an important biological source of Reactive Oxygen Species (ROS) contribute to oxidative stress and then to many diseases such as inflammation, atherosclerosis, cancer, aging, etc. It is an enzyme that plays a key role in hyperuricemia, catalyzing the oxidation of hypoxanthine to uric acid and xanthine. Hyperuricemia CHF patients, XO inhibition with allopurinol improved peripheral vasodilator capacity and blood flow to both local and systemic [13]. Treatment with allopurinol to prevent the production of reactive oxygen metabolites [14] and cause a potent inhibitory effect XOD with an IC50 of 8.4 pg / ml. XOD inhibitors are known to be therapeutically useful for the treatment of gout, hepatitis and brain tumors [15]. But we know that, according to Nuhu's work, flavonoids have been reported as potent inhibitors of xanthine oxidase [16].

Moreover, previous results have shown that the consumption of mushrooms may be beneficial to the antioxidant and XOD inhibitory protection system in the human body against oxidative damage and other complications [16].

Materials and Methods

Plant material

Fruiting bodies of Amanita muscaria, Amanita ceasarea, Agaricus campestris and Agaricus silvicola were collected in France during autumn 2005. Voucher specimens of these were identified and deposited at the Botanical laboratory of the Faculty of Pharmacy in Toulouse.

Preparation of extracts

Ground fruiting bodies from lyophilized fungi, Amanita muscaria, Amanita ceasarea, Agaricus campestris and Agaricus silvicola, (50 g), were extracted with boiling water (0.5 L) during 30 min. Extract yields of the 4 mushrooms were 29.7%, 37.3%, 42.4% and 39.3% (w/w), respectively.

Thin layer chromatography (Ph. Eur. V.6.20.2)

TLC was developed on Silica gel plate F254 (5-40 microns) with mobile phases. 10 µl of each aqueous extract were loaded on the TLC plate and developed up to 8-10 cm in CHCl3: MeOH: H2O 64:50:10 and dried in air. For detection, the TLC plates were stained with 2.54 mM DPPH solution in methanol, visualized under visible light. White spots on the purple background represent the inhibition when DPPH was sprayed.

Chemicals

All chemicals used were of analytical reagent grade. All reagents purchased from Sigma-Aldrich, Fluka (Saint-Quentin France).

Total amount of phenolic compounds

The total phenolic amount of each extract was determined by the Folin-Ciocalteu method [17]. A diluted solution of each extract (0.5 mL) was mixed with Folin-Ciocalteu reagent (0.2 M, 2.5 mL). This mixture was allowed to stand at room temperature for 5 min and then sodium carbonate solution (75 g/L in water, 2 mL) was added. After 1h of incubation, the absorbance was measured at 765 nm against blank using a Helios spectrophotometer (Unicam, Cambridge, UK). A standard calibration curve was plotted using Gallic acid (0-300 mg/L). Results were expressed as g of Gallic acid equivalents (GAE)/Kg. of dry mass.

Condensed tannin content

Catechin and proanthocyanidin reactive with vanillin were analyzed by the vanillin method [18]. One milliliter (1 mL) of each extract solution was mixed in a test tube with 2 mL of vanillin (1% in 7 M H_2SO_4) in an ice bath. Then the mix was incubated at 25°C. After 15 minutes, the solution absorbance was read at 500 nm. Concentrations were calculated as g catechin equivalents (CE)/Kg dry mass from a calibration curve.

Total flavonoids determination

The total flavonoids were estimated according to the Dowd method as adapted by Arvouet-Grand [19]. A diluted methanolic solution (4 mL) of each extract was mixed with a solution (4 mL) of aluminum trichloride ($AlCl_3$) in methanol (2%). The absorbance was read at 415 nm after 15 minutes against a blank sample consisting of a methanol (4 mL) and extract (4 mL) without AlCl3. Quercetin was used as reference compound to produce the standard curve, and the results were expressed as g of quercetin equivalents (QE)/Kg of dry mass.

Determination of total anthocyanin content

Total anthocyanin content was measured with the pH differential absorbance method, as described by Cheng and Breen [20]. Briefly, absorbance of the extract was measured at 510 and 700 nm in buffers at pH 1.0 (hydrochloric acid-potassium chloride, 0.2 M) and 4.5 (acetate acid-sodium acetate, 1 M). The wavelength reading was performed after 15 minutes of incubation. Anthocyanin content was calculated using a molar extinction coefficient (ε) of 29600 (cyanidin-3-glucoside) and absorbance of $A = ((A_{510} - A_{700})_{pH\ 1.0} - (A_{510} - A_{700})_{pH\ 4.5})$. Results were expressed as mg cyanidin-3-glucoside equivalent (C3GE) /Kg of dry mass.

Free radical scavenging activity: DPPH test

Antioxidant scavenging activity was studied using 1,1-diphenyl-2-picrylhydrazyl free radical (DPPH) as described by Blois [21] with some modifications; 1.5 mL of various dilutions of the test materials (essential oil or plant extracts) were mixed with 1.5 mL of a 0.2 mM methanolic DPPH solution. After an incubation period of 30 minutes at 25°C, the absorbance at 520 nm was recorded as $A_{(sample)}$, using a Helios spectrophotometer (Unicam, Cambridge, UK). A blank experiment was also carried out applying the same procedure to a solution without the test material and the absorbance was recorded as $A_{(blank)}$. The free radical-scavenging activity of each solution was then

calculated as percent inhibition according to the following equation:

$$\% \text{ inhibition} = 100 (A_{(blank)} - A_{(sample)}) / A_{(blank)}$$

Antioxidant activity of essential oil or extracts was expressed as IC50, defined as the concentration of the test material required to cause a 50% decrease in initial DPPH concentration. Ascorbic acid was used as a standard. All measurements were performed in triplicate.

ABTS radical-scavenging assay

The radical scavenging capacity of the samples for the ABTS (2,2'-azinobis-3-ethylbenzothiazoline-6-sulphonate) radical cation was determined as described by Re et al. [22]. ABTS was generated by mixing a 7 mM of ABTS at pH 7.4 (5 mM NaH_2PO_4, 5 mM Na_2HPO_4 and 154 mM NaCl) with 2.5 mM potassium persulfate (final concentration) followed by storage in the dark at room temperature for 16h before use. The mixture was diluted with ethanol to give an absorbance of 0.70 ± 0.02 units at 734 nm using a spectrophotometer. For each sample, diluted methanol solution of the sample (100 µL) was allowed to react with fresh ABTS solution (900 µL), and then the absorbance was measured 6 minutes after initial mixing. Ascorbic acid was used as a standard and the capacity of free radical scavenging was expressed by IC_{50} (mg/L) values calculated, denoting the concentration required to scavenge 50% of ABTS radicals. The capacity of free radical scavenging IC_{50} was determined using the same previously used equation for the DPPH method. All measurements were performed in triplicate.

XOD Inhibitory Activity Determination

The measurement of the XOD inhibitory activity was carried out according to the method of Wu et al. [23] with slight modifications. First, 798 µL of 0.1 unit XOD in buffer (200 mM sodium pyrophosphate/ HCl, pH 7.5) and 2 µL of the test extracts or compounds in water were mixed at 25-27°C for 5 min. The control group contained no test agent. The reaction was started by adding 200 µL of 0.6 mM xanthine in double distilled water to the mixture. The reaction mixture was incubated at ambient temperature. Finally, the absorption increments at 295 nm, which indicated the formation of uric acid, were determined every min up to 10 min. Allopurinol was used as a positive control. Five replicates were made for each test sample. The percent inhibition ratio (%) was calculated according to the following equation: % inhibition = [(rate of control reaction - rate of sample reaction)/rate of control reaction] x 100.

Statistical analysis

All data were expressed as means ± standard deviations of triplicate measurements. The confidence limits were set at $P < 0.05$. Standard deviations (SD) did not exceed 5% for the majority of the values obtained.

Results and Discussion

Given our results, the 4 fungal powders Amanita muscaria, Amanita Caesarea, Agaricus campestris and Agaricus silvicola have antioxidant activity as shown by TLC (Figure 1) and Table 2. On this TLC, we find that qualitatively Agaricus campestris is the most active fungus, which will be supported by the results of spectrophotometric assays (Figure 3). Figure 2 allows to visualize the chemical profile of fungal aqueous extracts in four molecules families.

Wild mushrooms, the last three are edible and appreciated, could see their initial use food valued. However, without neglecting the synergy of bioactive compounds present in these extracts, these results are encouraging to explain and corroborate the food use of fungal fruiting bodies in the prevention and treatment of oxidative damage.

Figure 3 shows the fungal extracts chemical potential. We find that Agaricus silvicola although rich in polyphenols (36.6 ± 0.24 g Gallic acid equivalent / Kg dry weight), has a very low concentration of flavonoids (1.09 ± 0.15 g quercetin equivalent (QE) / kg dry weight) (Table 1) that can certainly explain the inflection of the curve and its very low antioxidant activity with ABTS (IC50 of 188.9 ± 3.4 mg/mL). It is interesting to note that for fungus aqueous extracts the DPPH assay gives generally higher results than ABTS assay, except for Agaricus silvicola. There may be a correlation with the concentration as well as with the nature of extracted flavonoid. For four fungi studied, we find that the XOD inhibitory activity (Table 2) is correlated with the concentration ratio of flavonoids / polyphenols ((18.9 ± 0.21 g quercetin equivalent / kg dry weight)/(25.9 ± 3.6 g Gallic acid equivalent / Kg dry weight)) with maximum activity in Agaricus campestris which has also the maximum concentration of tannins (5.6 ± 0.12 g Catechin equivalent / Kg). Agaricus campestris and Agaricus silvicola are most active in the DPPH and also in the inhibition of XOD.

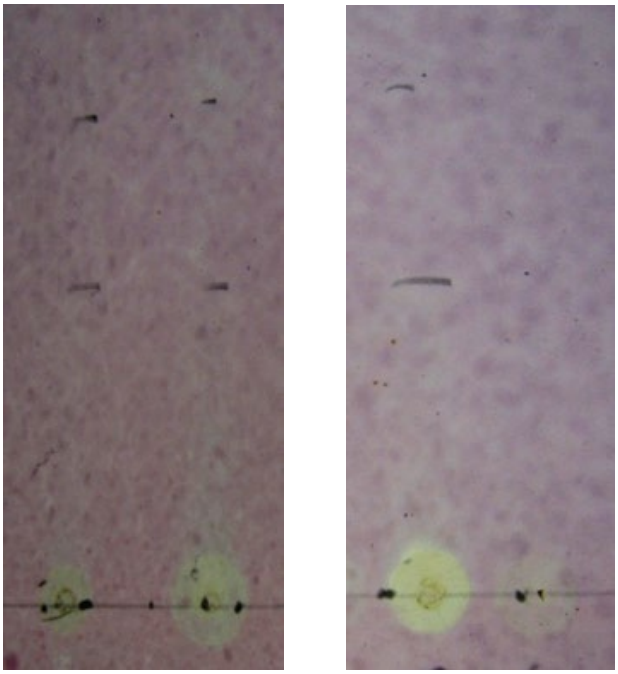

Figure 1. Screening for antioxidant properties using the TLC assays method. TLC plates stained with 2.54 mM DPPH solution in methanol, visualized under visible light. White spots on the purple background represent the inhibition when DPPH was sprayed. A total of 10 µl samples were loaded on the TLC plate, and developed in CHCl3: MeOH: H2O 64:50:10. Fungal aqueous extracts: 1- Amanita caesarea; 2- Amanita Muscaria; 3- Agaricus campestris; 4- Agaricus silvicola – (50 g/0.5L H2O) fungi.

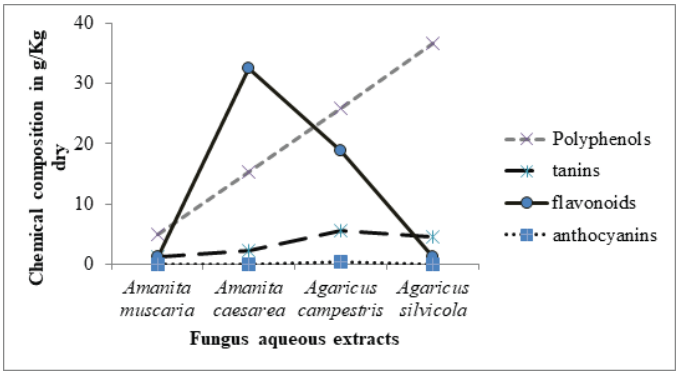

Figure 2. Chemical composition of fungus aqueous extracts

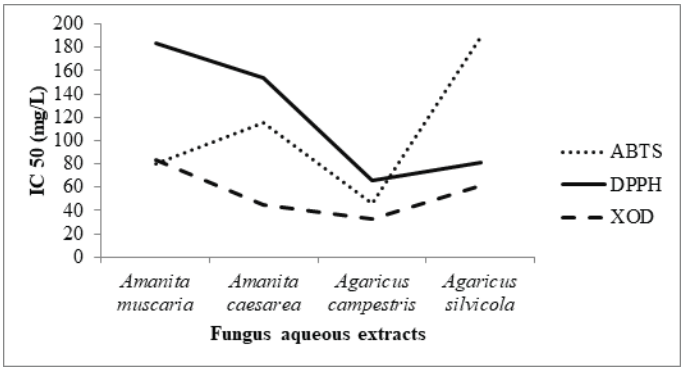

Figure 3. Activities of fungus aqueous extracts

Table 1. Chemical composition of fungi aqueous extracts

Aqueous extracts	Polyphenols (eq Gallic acid)[a]	Tannins (eq Catechin)[a]	Flavonoids (eq Quercetin)[a]	Anthocyanins (eq cyanindin)[b]
Amanita muscaria	4.86 ± 0.24	4.86 ± 0.24	1.25 ± 0.11	4.86 ± 0.24
Amanita caesarea	15.23 ± 0.02	15.23 ± 0.02	32.6 ± 2.56	15.23 ± 0.02
Agaricus campestris	25.9 ± 3.6	25.9 ± 3.6	18.9 ± 0.21	25.9 ± 3.6
Agaricus silvicola	36.6 ± 0.24	36.6 ± 0.24	1.09 ± 0.15	36.6 ± 0.24

Table 1. A fungus aqueous extracts chemical activities

	ABTS assay IC_{50} (mg/L)	DPPH assay IC_{50} (mg/L)	XOD assay IC_{50} (mg/L)
Amanita muscaria	79.69 ± 1.9	183.46 ± 1.9	83.36 ± 2.5
Amanita caesarea	115.09 ± 3.1	153.26 ± 1.26	45.1 ± 1.2
Agaricus campestris	45.6 ± 0.8	65.3 ± 1.59	32.78 ± 1.09
Agaricus silvicola	188.9 ± 3.4	80.9 ± 1.7	61.55 ± 3.1
Vit C	1.8 ± 0.1	4.5 ±0.3	
Allopurinol			7.5 ± 0.9

For the ABTS assay, in light of these preliminary results, we can hypothesize that the antioxidant activity found in edible mushrooms could be a consequence of the inhibition of XOD, playing a protective role in the cardiovascular system, in the characteristic system the southwest of France. Indeed, the synergy between wine and diet rich in fruits, vegetables and mushrooms would be an interesting way to explain the famous French paradox. It remains a topic that the scientific community trying to understand. Our work aims to illustrate and emphasize the importance of fungal natural substances in this vast subject. The inhibitory activity of the aqueous extracts XOD suggests that fruiting fungi studied can potentially be used as a readily available source of natural antioxidants and inhibitors of XOD with promising potential in the prevention of related diseases to ROS.

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