PRELIMINARY PHARMACOBOTANICAL STUDY ON CULTURED PLEUROTUS SPECIES

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ABSTRACT. *Pleurotus ostreatus* – the oyster mushroom, grows worldwide in the wild on deciduous trees in shelf-like clusters. The most important quantity of this mushroom comes from controlled cultures, grown on farms, from mycelia cultivated on liquid or solid substrates. Taking into consideration its highly appraised nutritional value as well as the therapeutical properties of *Pleurotus* species, the aim of the present study is a preliminary analisys to establish the morphological and qualitative parameters and the content in protein and polysaccharides for the basidiocarp as a whole, and separately for the stipes

Keywords: *Pleurotus ostreatus* L., cultured, qualitative and quantitative determination

INTRODUCTION

Many types of edible and poisonous mushrooms have long been used worldwide, but especially in the Middle East, for medicinal purposes. At present times, 80-85% of medicinal mushrooms products are obtained from cultured mushrooms. For the purpose of extracting the pharmacologically valuable compounds, generally whole mushrooms, extracts, or isolated compounds can be used. These compounds can be obtained from mushrooms growing on farms or in their natural habitat, from mycelia cultivated on liquid or solid substrates. (Wasser 1999, Babitskatya 1999, Mateescu 1983). Pleurotus is one of the easiest mushrooms to cultivate, most often on straw or on logs with sawdust. This species has become one of the most well known edible mushrooms, having a pleasant odour and taste. Nowadays about 1 million tons of Pleurotus mushrooms are produced, especially in China, Germany and the USA. In the world top of the mushrooms cultivated for culinary purposes, Pleurotus comes second after *Agaricus campestris* (*Agaricus bisporus*) (Weasel 1998). The most cultivated species among the genus Pleurotus are: *Pleurotus ostreatus* (figure 1), *Pleurotus florida, Pleurotus cornucopiae.* The most important goals of the cultivators are to ensure a superior quality and a homogeneity of composition for each harvest (Burzo 1980, Mateescu 1983).

Fig. 1 Pleurotus ostreatus

MATERIAL AND METHODS

The vegetal product subjected to analysis is represented by the fruiting body of the species *Pleurotus ostreatus* obtained from controlled cultures: I/4 series and II/4 series. The two series represent the species harvested in the first flush, on the fourth day, and on the fourteenth day from the basidiocarp formation period. The methods employed in determining the identity and the quality of a vegetal product subjected to analysis are the qualitative methods: macroscopic, microscopic, microchemical, referred to in the specialty books (Fasidi 1994, Parv 1996, R.F.X 1998).

Macroscopic examination of the basidiocarp

The macroscopic examination determines the main morphological characteristics of the fruiting bodies subjected to analysis: colour, fracture, odour, taste. The colour of the fruiting bodies is varied, being one of the main criteria in the macroscopic identification of the

species. It differs from one species to another, and it is determined by the pigment in the cytoplasm and in the hyphal walls. Depending on the water solubility of the pigments, the fruiting bodies of some mushrooms change their colour on rainy days. Also, when touching, breaking or crushing, in contact with the air, the flesh or the external surface of some mushrooms change their colour to blue, green, yellow, brown or red as a result of oxidation reactions of some of the substances contained. The smell and the taste are characteristic. The taste is perceived differently: immediately or after a long while. The main characteristic of the fruiting body is spore production. At a certain stage of development a fertile area is individualized on the fruiting body – the hymeneal region which produces the basidia with the basidiospores. A palisade layer of prolonged cells is being differentiated, from which the basidia develop, and in which at the beginning the two adjacent nuclei form 4 exogenous spores disposed at the top of the sterigmata – the basidiospores.

Table I presents the results of the microscopic examination for the I/4 and II/4 series.

Table 1

MACROSCOPIC EXAMINATION OF THE BASIDIOCARP

Fruiting body	Blue-grey, brown or black
Pileus	The diameter of the pileus is 10 – 15 cm, the colour is whitish, greyish then rust-coloured or slightly green.The cap cuticle is dusty on dry weather and sticky on humid weather. The pileus presents a hymeneal layer formed of pink or dark-coloured gills, forming rays. It is bell-shaped, with dark-coloured spots at maturity. At maturity, the hymeneal layer is opened through the pores from which the characteristic spores will be delivered.
Stipe	The stipe is ovoid, globose, thickened at base with a red, blood-like network at the upper part. The flesh is white or white-yellowish. At some species the flesh is reddish. At young species the stipe is convex, and it becomes elongated without a volva. The coulour of the stipe is white with characteristic dotted ornamentation, acting as a recognition criterion.
Fracture	After being fractured, in contact with air the white colour of the pileus turns to red and then to blue.
Odour	Characteristic
Taste	Immediately perceived

Microscopic examination of the basidiocarp

The examination was carried out using an optical microscope Ultimate digital model MFL-85.

1. Species identification according to taxonomic position, using specific reagents

Melzer's reagent preparation

0,5 g iodine and 1,5 g potassium iodide are dissolved in 20 ml water and 20 ml chloral hydrate. The components are boiled in a Soxhlet installation for 40-60 minutes over a low flame, followed by filtration. The colour reaction of the spores with Melzer's reagent represents one of the main criteria in species determination. When identifying genera the colour of the spore dust is considered, which can be obtained by placing under a glass bell a sporocarp with the fertile region facing downwards on a black sheet of paper. The spores are laid down after a few hours, a sporogram being thus obtained.

Results: The spores obtained from *Pleurotus ostreatus* cultures are long, unicellular, light yellow. With Melzer's reagent the same blue coloration was obtained. The sporogram is identical. The elongated hyphae are fragmented, appearing as septate tubes (figure 2). The spores are long unicellular with dimensions ranging between 0,2-0,5 cm, the colour is white-yellowish, ornamentation being characteristic.

Fig. 2 Pleurotus ostreatus, unicellular spores, elongated and fragmented hyphae

Microscopic characterization of the analyzed series I/4 and II/4

Microscopic examination of the flesh distinguishes several types of hyphae:

- fundamental hyphae or inflated long-celled hyphae
- generative hyphae or growing hyphae with side clamps

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- hyphae with a rich content of reserve substances

-oliferous hyphae , rich in oils and odoriferous substances

- skeletal hyphae, with thick, woody or suberous walls

The fruiting bodies, represented by the mycelial hyphae, are differentiated at the two series by the density of the lax texture. Their dimensions range from a few millimeters to 1-2 cm. A secondary mycelium composed of septate hyphae with binuclear cells, with ramifications and strings is identified. The sterigmatae bear the basidiospores with 4 elongated basidia, of white colour on the coloured substrate represented by the primary mycelium, with numerous ramifications.

Determination of dry substance content and of residuum by calcination

The Romanian Pharmacopoeia Xth edition sets out the working method for establishing the mass of volatile compounds of any nature in the vegetal product subjected to analysis. The loss is calculated according to certain conditions of temperature, pressure and time. The results obtained are expressed in grams and are calculated in percents (% m/m).

Determination is carried out in measuring vials whose diameter is chosen so that the layer is 1 cm thick. The measuring vial is brought to constant mass under the same conditions as the determination itself.

The samples to be analyzed are collected from the two series and are represented by the whole fruiting body and separately by the stipes, freshly harvested. 5 gram samples of the product, grounded to small fragments are measured on the analytical balance.

Determination procedure

The measuring vial with the working sample is kept in the drying oven at 105ºC for 3-4 hours, cooled in a dessicator and measured. The drying process is furthered for periods of time of one hour, followed by cooling in the dessicator and measured until constant mass.

Determination of residuum by calcination

The residuum by calcination is the residuum obtained by the calcination of an organic substance. In the case of vegetal products the residuum obtained is referred to as ash. The results obtained are expressed in grams and calculated in percentages (% m/m).

Working technique

The porcelain capsule used for the determination of the residuum by calcinations is brought to constant mass by maintaining it to the same temperature the calcination is to be carried out and it is cooled in the dessicator. 5 g samples are measured as follows: whole fruiting bodies harvested from the series I/4 and II/4, and fresh separate stipes from the same series.

The sample grounded to small fragments is measured in the porcelain capsule on the analytical balance and heated on the wire gauze in low flame till the removal of water and other volatile substances. Heating is continued on a chamotte triangle, gradually increasing the temperature of the flame; heating is continued until carbonization. The capsule is cooled in the dessicator and weighed. Calcination is carried on, for 15 minutes followed by cooling in the dessicator and measuring till constant mass. To the residuum obtained from calcination 2-3 ml 100g/l hydrochloric acid is added. The capsule is covered with a watch glass and heated in the water bath for 10 minutes. 5 ml of 70 ºC heated water is added – also used for washing the watch glass-and the content is filtered using a microporous filter. The precipitate is brought on the filter and washed with 70 ºC heated water until the reactions of the wash waters with the chloride ion are negative. The filter containing the precipitate is dried at 105ºC, placed in the initially used capsule and calcined to constant mass.

Determination of proteins

More than half of the dry mass of the mushroom is formed of proteins containing all the essential amino acids. The mycelium uses sugars, organic acids as carbon source and components resulting from decomposition of proteins such as peptones, amino acids, urea and ammonia as nitrogen source.

Material and methods

Determination of overall protein content was carried out using the gravimetric method for the two representatives of I/4 and II/4 series.

The gravimetric method of measuring the protein content is based on the property of proteins to precipitate with salts of heavy metals (Hg^{2+}, Ag^{+}, Pb^{2+}), organic solvents such as ethanol, methanol, acetone or acid reagents (trichloroacetic acid, picric acid, phosphomolybdic acid). Extraction from the vegetal material can be performed using water, hydroalcoholic or saline solutions, phosphate buffer. The vegetal material was carefully treated not to damage the properties of the proteins.

Preparation of samples

Samples 1 and 2- whole fruiting bodies from series I/4 and II/4. Drying was performed under natural conditions keeping them from direct sunlight.

Samples 3 and 4– separate dry stipes from the two series

Working conditions

- extraction temperature 4-5ºC (refrigerator); separation by filtration; vacuum drying, maximum temperature 30 ºC; extraction solvent: phosphate buffer pH=7,4; precipitation solvent: methanol; precipitation time: 48 hours.

Working method

The vegetal materials are brought to the optimal grinding degree (sieve IV). 2,0 g samples are weighed and transferred to an adequate container, then 80 ml phosphate buffer pH=7,4 is added and stirred. The container is kept in the refrigerator at 4-5 ºC for 48 hours. It is stirred periodically then filtered through cotton wool. The residuum is washed twice with 10 ml phosphate buffer and 10 ml methanol is added by stirring. The mixture is kept at rest in the refrigerator for 24 hours followed by quantitative filtration, the filter is washed with methanol twice (maximum 10 ml). The filter with the precipitate is dried in the vacuum drying oven. The quantity of proteins resulted is determined by weighing. The obtained value is determined using the formula:

$$\text{proteins \%} = \frac{M1 - Mo}{a} \text{ x } 100$$

where:
M1= mass of filtrate with precipitate
M0= mass of filter
a= quantity of working vegetal material

Extraction of polysaccharides

There is a certain resemblance between the various methods employed for the extraction of antineoplastic polysaccharides from mushrooms. In the first stage, the dried powder from the mushrooms or from the mycelium is gradually heated in 80 % alcohol to extract and remove the substances with low molecular mass. Several gross fractions are obtained from the alcoholic residuum:

I. by water extraction at 100 ºC for 3 hours
II. 1% ammoium oxalate at 100 ºC for 6 hours
III. 5% sodium hydroxide at 80 ºC for 6 hours

The most effective method for extracting D glucan from *Pleurotus ostreatus* uses ethanol precipitation followed by lyophilization. Using a carbohydrate chromatography column the purity of the extract could be established, which is 87,5 % lentinan. The commercial value of this new method is of great importance as as it is more rapid, economically affordable and effective. The samples subjected to analysis are species of *Pleurotus* from I/4 and II/4 series. Determination of overall polysaccharide content was carried out at the Biology laboratory, University of Pecs, employing the *Yap* and *Neugemann* separation method.

RESULTS AND DISCUSSIONS

As a result of determinations the chemical content of the separate parts of *Pleurotus ostreatus* fruiting body could be established. The results are presented in table II and histogram 3.

In the whole fruiting bodies, for both series, a quantity of over 40 % of protein from the overall dry substance was determined. The species harvested in the first flush, on the fourth day of basidiocarp formation contain with 4 % more protein than those from the second flush, harvested on the 14th day. For the separate stipes, the determined values represent ¼ of the overall content of protein, for both series.

The values obtained certify the homogeneity of the cultures and the high nutritional value of the analyzed *Pleurotus* species.

Table 2

CHEMICAL CONTENT OF PARTS OF PLEUROTUS OSTREATUS

Analyzed vegetal material	Dry substance % m/m	Water and volatile compounds % m/m	Residuum by calcination % m/m	Proteins % m/m	Overall polysaccharide content % mg/g
Fruit I/4	9,842	90,158	6,321	46,22	325
Fruit II/4	9,456	90,544	6,073	41,13	318
Stipes I/4	7,230	92,770	4,643	11,55	-
Stipes II/4	7,441	92,559	4,779	10,28	-

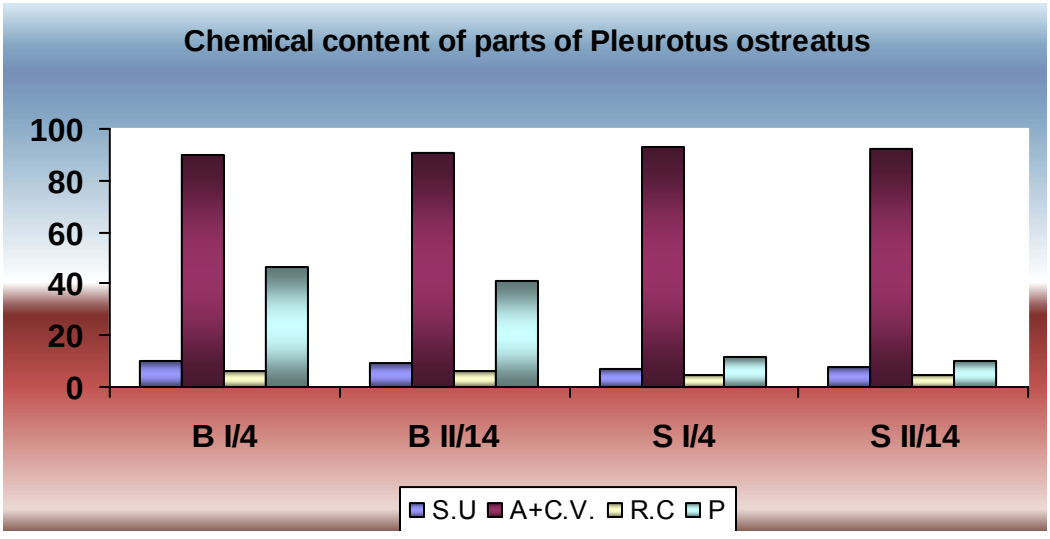

Fig. 3 Average chemical composition established for analyzed Pleurotus series
A.S. -dry substance; A+C.V –water and volatile compounds;
R.C. -residuum by calcination, P.– proteins

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CONCLUSIONS

Determination of chemical composition of the fruiting bodies involved:

Determination of content of dry substance, the values obtained ranging between 7,230% m/m and 9,842 % m/m, being lower for the stipes compared to the basidiocarp.

Determination of residuum by calcination (ash) led to lower values of 4,643 % m/m compared to the basidiocarp.

Determination of protein content certified the fact that more than half of the dry weight of the mushroom consists of proteins with all the essential amino acids. The mycelium uses sugars, organic acids as carbon source and components resulting from decomposition of proteins such as peptones, amino acids, urea and ammonia as nitrogen source. The maximum values determined for the basidiocarp were 46,22 % m/m and 11,55 % m/m for the stipes. Separately for the stipes, the determined values represent ¼ of the overall content of proteins. The values obtained certify the homogeneity of the cultures and the high nutritional value of the analyzed *Pleurotus* species.

Extraction of polysaccharides employing a modern, easy to use, highly efficient method facilitated obtaining values of 318- 325 (mg/100g) overall purified polysaccharides. It can be stated that the period of harvesting does not significantly influences the content of overall polysaccharides, responsible for the antineoplastic and immunostimulant effects.

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