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Determination of the antioxidant activity of Oyster mushrooms from U.P. VIII Răchita

Petru DRAGOMIR[1], **Maria DOBOȘ**[1], **Camelia TULCAN**[1], **Mihaela MOATĂR**[1*], **Dorin CAMEN**[2], **Roberta TRIPON**[1], **Alexandra DRAGOMIR**[1], **Carolina ȘTEFAN**[1], **Alexandru PANICI**[1]

[1] University of Life Sciences "King Mihai I" from Timișoara, Faculty of Engineering and Applied Technologies, Department of Forestry, e-mail: petruioandragomir@usvt.ro, cameliatulcan@usvt.ro, mihaela.moatar@usvt.ro, roberta.tripon@usvt.ro, carolinastefan@usvt.ro, alexandrupanici@usvt.ro
[2] University of Life Sciences "King Mihai I" from Timișoara, Faculty of Engineering and Applied Technologies, Department of Genetic Engineering, e-mail: dorincamen@usvt.ro

* Corresponding author: mihaela.moatar@usvt.ro

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Abstract
Oyster mushrooms (*Pleurotus ostreatus Jacq. P. Kumm.)* from forest of UP VIII Răchita were investigated for determination of polyphenol content (Folin-Ciocâlteu method), determination of total flavonoids (sodium nitrite method) and evaluation of antioxidant activity using the DPPH method. The highest total polyphenol value was obtained for the methanol extract of *Pleurotus ostreatus* subjected to the ultrasound procedure, respectively 14.74 ± 0.20 μgGAE/g, followed by the ethanol extract obtained by shaking (14.62 ± 0.04 μgGAE/g) and ultrasound (14.50 ± 0.09 μgGAE/g). Whilst the lowest value, 13.90 ± 0, 03 μgGAE/g was indicated by the methanol extract obtained by shaking. The minimum value was indicated by the extract of *Pleurotus ostreatus* in methanol obtained by ultrasound, this being followed by the same methanol extract, nonetheless obtained by shaking with a value of 133.77 ± 0.57 μg/ml. The highest percentage of inhibition was recorded for the methanol extract of *Pleurotus ostreatus* subjected to the stirring technique, having a value of 29.69%. This was followed by the methanol extract obtained by ultrasound with a PI of 25.65%. For the other two ethanol extracts, the percentages did not exceed 20%, respectively 18.78% for the extract in ethanol obtained by stirring and 17.09% for the one obtained by ultrasound.

Keywords: phenolic content, flavonoid content; antioxidant activity, oyster mushrooms.

Introduction

Beech sponges are basidiomycete fungi characterized by an eccentric foot. They appear in October – February, both in plain and mountain areas, on the rotten wood material of the gazelles. They are good for consumption, containing important amounts of protein substances, lipids and vitamins and are widely used for biotechnological interest [14].

Phenolic chemicals are phytonutrients, powerful free radical scavengers and antioxidants found in mushrooms [8, 9]. This extremely complex collection of metabolites, which includes tannins, lignin, stilbenes, phenolic acids, and flavonoids, all have the property of having one or more aromatic rings with hydroxyl groups in their structure [8, 5].

Different growth techniques have been effectively adopted to boost the nutritional value of mushrooms, since the lack of micronutrients is linked to health problems [3, 4, 7].

The determinations aimed at quantifying the antioxidant properties of *Pleurotus ostreatus* or alcoholic extracts were channelled on the determination of total polyphenols, total flavonoids and the percentage of inhibition of colour development (DPPH, Trolox equivalent), the latter being directly proportional to the antioxidant capacity of the extract.

Material and Method

The study material comes from U.P. VIII. Răchita, O.S. Teregova, the land of the Banat Mountains, in the basin of the Timis River [15].

The main objective of the study is to determine the antioxidant activity of beech sponges by:

1. Determination of polyphenol content (Folin-Ciocâlteu method).

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2. Determination of total flavonoids (sodium nitrite method).
3. Assessment of antioxidant activity by the DPPH method.

For the preparation of the sample to be analysed, approximately 10 g of the sample prepared for analysis was weighed in the ampoule, with an accuracy of 1 mg [A.1, A.2]. The vial was placed into the 103°C hold and allowed to dry for 4 h ± 0.1 h [A.3], after the temperature of the flask has again reached 103°C. After the 4 hours had passed, we removed the vial from the chamber and allowed it to cool to room temperature in the exicator, then weighed exactly 1 mg.

Obtaining the alcoholic extract:

To obtain the alcoholic extract, the following steps were taken:

- Drying of plant material as indicated by ISO 1573:1980 [A.3].
- Grinding of the plant product to obtain fine dust [A.4].
- Weighing 0.200 ± 0.001 g of powder obtained in two different containers, working in parallel [A.5];
- Preparation of a 70% methanol solution followed by the steps described in ISO 14502-1:2005 (addition of 5 ml of 70% methanol at a temperature of 70°C above the plant material, vortexing of the plant-methanol preparation [A.6], ultrasonating or stirring it* [A.7, A.8], centrifugation at 3500 rpm 10' [A.9] followed by collection of supernatant and bringing to a final volume of 10 ml with the specific solvent [A.10, A.11].
- For alcoholic extracts, two physical methods were used to facilitate the extraction process, namely agitation (Shaker Panasonic MIR-S100) and ultrasonication on the bathroom (bath ASonic PRO50).

Determination of total polyphenols by Folin-Ciocâlteu method:

Colorimetric reactions were widely used in UV/VIS spectrophotometric determinations because they are fast, easy to perform and applicable in various fields of work. However, a very important aspect in these determinations is the use of a reference material to which the results obtained from the determination will be related.

Polyphenols, secondary products of plant metabolism, could react with specific redox reagents, with the formation of a blue complex that can be discerned by spectrophotometry in the visible field. Such a reagent is also the most specified, Folin-Ciocâlteu, representing a mixture of phosphomolibdat and phosphotungstat [1].

The oxidation-reduction reaction consists of the transfer of electrons from phenolic groups to phosphomolibdic acid and phosphotungstic compounds in an alkaline environment [12]. Sodium carbonate (RN 223-3) is the compound that provides alkalinization of the environment, extending the pH value to a value equal to or greater than 10 [11]. This alkalinization is an essential condition for a correct reaction of phenolic groups with the Folin-Ciocâlteu reagent [6, 12]. As a result of the reaction, phenolate ion groups are formed which lead to the reduction of the acid compounds of the reagent. This mechanism is confirmed by changing the original colour from light yellow to blue (colour indicating reduced acid status), with a intensity directly proportional to the number of reactive phenolic groups [6, 11].

In order to determine the total polyphenol content, the method described by Tamas-Krumpe Octavia and his collaborators was chosen in 2010 (Tamas-Krumpe, O. Maria., et al., 2010) "the analysis of the biologically active compounds content and the antioxidant potential of some Romanian Polyfloral Honey samples", Filodiritto Editore – proceedings, 2010), procedure consisting in the use of the Folin-Ciocâlteu 0.2 N reagent and sodium carbonate (Na2CO3) and the use of gallic acid in different concentrations as standard.

Work method:

- 25 µL of each 1:100 diluted alcoholic extract (ISO 14502-1:2005) [A.12] pipetted into a microplate (TPP®) with 96 wells.
- 125 µL of Folin-Ciocâlteu reagent and 100 µL Na2 CO3 were pipetted over the samples to be analyzed (alcoholic extracts).
- The resulting complex was kept at room temperature in a place away from light for 60 minutes (ISO 14502-1:2005), developing a blue colouring [A.13].
- The optical densities for the analysed extracts were spectrophotometric quantified at a wavelength of 765 nm using a Tecan Infinite M1000 Pro spectrophotometer [A.14].

To achieve the standard curve, gallic acid was chosen as standard with eight different concentrations, namely: 0 µg/ml, 3.9 µg/ml, 7.8 µg/ml, 15.62 µg/ml, 31.25 µg/ml, 62.50 µg/ml, 125 µg/ml and 250 µg/ml, being pipetted under the same conditions as samples. The results were reported at the concentrations on the standard curve.

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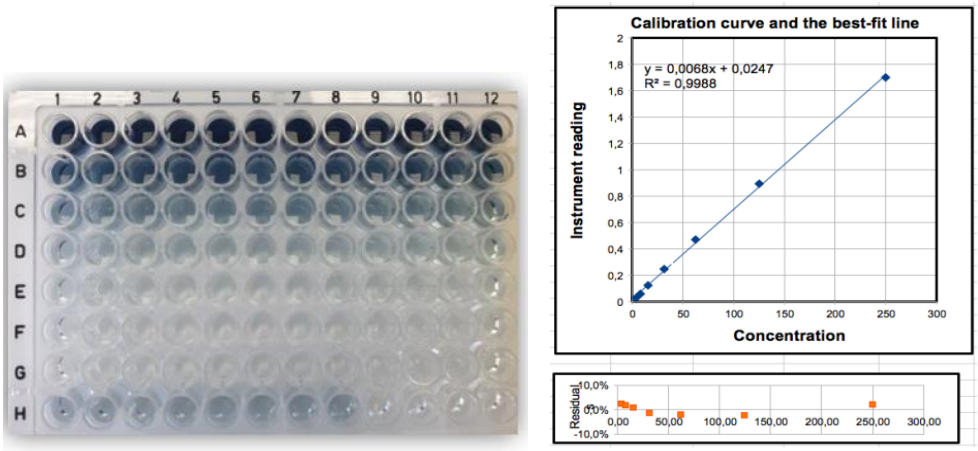

Figure 1. Standard curve for gallic acid standards (y=mx+b)

Following the graphical representation of optical densities values according to concentration, the calibration curve described by the equation y=mx+b was obtained with a correlation coefficient (r 2) is equal to 0.9988. For each extract, the values of the optical denistations obtained after the blank value has been extracted have been entered into the concentration calculation application using the linear function of the calibration curve.

Determination of the total flavonoid content by the sodium nitrite method:

Flavonoids, such as polyphenols, are secondary products of metabolism in different plant organs. They exhibit remarkable antioxidant activity, dependent on the number of hydroxyl groups in their structure [2]. The spectrophotometric determination method based on the formation of the aluminum complex is the most used method for the analysis of the flavonoid content in plant matrices.

The reaction takes place in the presence of the$NaNO_2$ in an alkaline environment.

At the base of the reaction is the nitration mechanism of any aromatic ring that has in its structure a catecol group with three or four sterile positions.

After the addition of al (III), a yellow complex is formed, which when the NaOH is added turns red. The absorbance value of this complex can be read spectrophotometric at a wavelength equal to 510 nm.

As for the choice of the reference standard, catechin is preferred in the literature, but routine or quercetin are also very often used [7]. Regarding this study, the determination of the total flavonoid content in the alcoholic extract was carried out after the next sequence of steps and is based on the method described by S. Al-Matani and others in 2015 [1].

Working method:

- 500 µl of each sample was pipetted into 5 ml volumetric flasks.
- 150 µL NaNO2 (5%) freshly prepared was added, and the formed mixture was kept for 5' at room temperature.
- After the 5', 250 µL AlCl3 (2%) was pipetted at 6', at room temperature.
- After the pipetting of the aluminum solution, the yellow complex was formed.
- Over the newly formed complex were added 250 µL NaOH, the fraction time being 10'.
- Following this reaction, the red-brick shade presented in the description of the mechanism was obtained.
- Before the10' expiration, samples were brought to a final volume of 5 ml with distilled water.
- The absorbance of the extracts was read spectrophotometrically at 510 nm with a Perkin Elmer Lambda 25 spectrophotometer.

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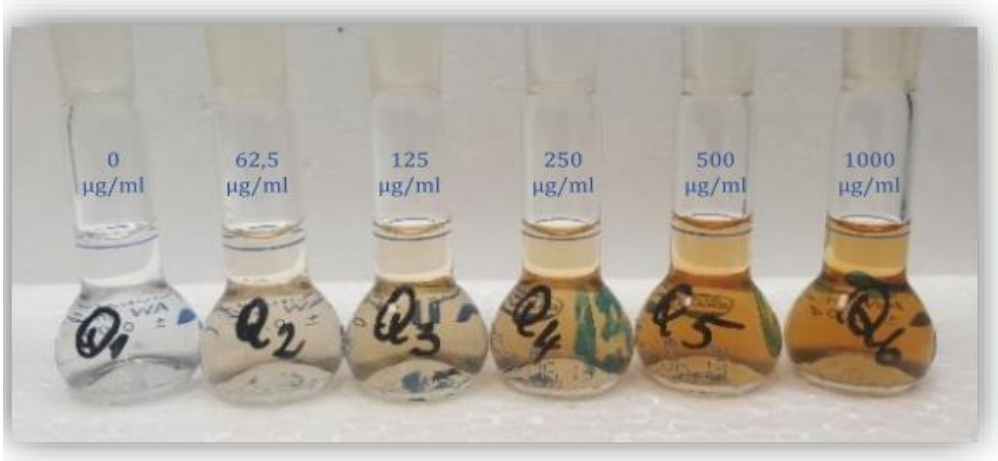

Figure 2. The six concentrations (Q1-Q6) of the routine standard

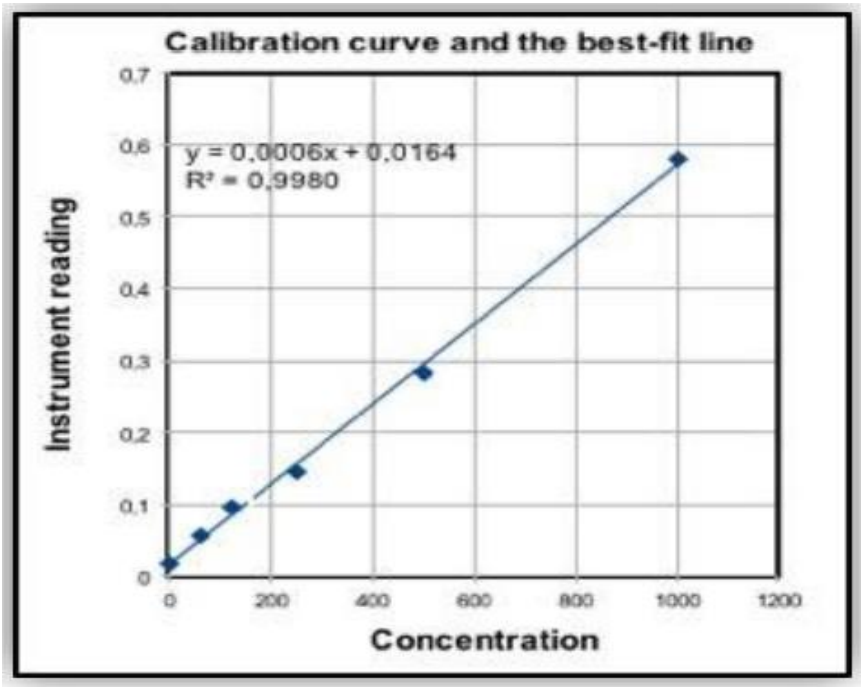

Figure 3. The standard curve

Following the introduction of optical densities values on the standard curve, a correlation coefficient (0.9980) was obtained.

Assessment of antioxidant activity employing the DPPH method:

Cells, tissues, and interstitial fluids possess remarkable protection systems that ensure the counteracting of all oxidative processes.

To maintain metabolic and functional integrity, in the aerobic environment, antioxidant protection is organized into three levels: Prevention, interception and repair. The term "total antioxidant capacity" means the association of the three concepts and is defined as "the cumulative action of all antioxidants in a tissue or liquid, thus representing an integrated parameter and not a mere sum of quantifiable antioxidants".

Studies to determine the total antioxidant capacity in human blood plasma samples indicated an increase in this parameter following consumption of flavonoid-rich and polyphenol-rich food.

These results underscored the hypothesis that the compounds mentioned above represent powerful antioxidants in vitro, having the ability to reduce the risk of developing chronic pathologies. In general, a multitude of fruits and plants contain several micro and macro-nutrients such as flavonoids, phenolic compounds or terpenes, compounds that influence both directly and metabolically this total antioxidant capacity [13].

As for antioxidant systems, there are two types of such systems:

- the first is the enzyme antioxidant system, including the three antioxidant enzymes catalase, superoxide dismutase and glutathione peroxidase.
- the second is the non-enzymatic antioxidant system and includes: Vitamin C, vitamin E, uric acid, glutathione, bilirubin, alpha-lipoic acid, as well as carotenoids.

All these enzymes, antioxidant molecules or macromolecules possess the ability to eliminate various compounds capable of preventing and limiting the oxidative stress caused by reactive oxygen species. The total level achieved by their activities is the total antioxidant capacity of the system.

In this study, the method is based on measuring absorbance at the wavelength of the specific maximum for DPPH, 515 nm. In the presence of an antioxidant, the absorbance recorded for DPPH decreases, the decrease in the value being proportional to the anti-radicalic activity, and the colour of the solution changes from purple to yellow/yellowish.

The more effective the compound is, the greater is the decrease. The obtained values are expressed in relation to a compound considered a reference antioxidant called Trolox (http://fitoplat.incdsb.ro/wp-content/uploads/2017/09/Protocol-1.3.pdf

The testing was carried out in accordance with the Protocol for the screening of the anti-radicalisation efficiency of phyto-compounds using DPPH (http://fitoplat.incdsb.ro/wp-content/uploads/2017/09/Protocol-1.3.pdf) and consisted of preparing a solution of DPPH 2,5x10-4 MOLL-1 in methanol, trolox 2,5x10-4 M in methanol, making blanks for both reagents and achieving the determination of total antioxidant capacity for alcoholic extracts.

Testing was performed in accordance with the protocol for screening the antiradical efficacy of phyto-compounds using *DPPH* (http://fitoplat.incdsb.ro/wp-content/uploads/2017/09/Protocol-1.3.pdf) and consisted of preparing a solution of DPPH 2,5x10-4 MolL-1 in methanol, trolox 2,5x10-4 M in methanol, performing blanks for both reagents and determining the total antioxidant capacity for alcoholic extracts.

Determining the blank for 2.2-diphenyl-1-picrylhydrazil (DPPH):

2 ml methanol was pipetted into a graduated container over which 1 ml 2.2-diphenyl-1-picrylhydrazyl 2,5x10-4 MOLL-1 was added with a reaction time of 3', followed by spectrophotometric reading of the 515 nm complex (Perkin Elmer Lambda 25).

Determining the blank for the Trolox:

1.9 ml methanol were pipetted into a graduated container over which 1 ml DPPH and 0.1 ml trolox 2,5x10 -4M were added with a reaction time of 3' followed by spectrophotometric reading of the complex formed at 515 nm (Perkin Elmer Lambda 25).

Determining the total antioxidant capacity for alcoholic extracts

1.9 ml methanol were pipetted into a graduated container over which 0.1 ml of alcohol extract and 1 ml of DPPH were added with a reaction time of 3' followed by spectrophotometric reading of the complex formed at 515 nm (Perkin Elmer Lambda 25).

The calculation of the antioxidant capacity presented as a percentage of inhibition of DPPH discoloration of extracts was made using the following formula:

$$I\% = \frac{Ablank - Aproba}{Aproba} \times 100$$

Where:

A_{blank} represents the maximum absorbance, read 3 minutes after adding 1 ml of DPPH, to a cell containing 2 ml methanol.

$A_{probă}$ the maximum absorbance, read 3 minutes after adding 0.1 ml of sample (approximately 2 mg/ml) to a cell containing 1 ml DPPH and 0.9 ml methanol.

Results and Discussion

The results of the determinations aimed at quantifying the antioxidant properties of alcoholic extracts of Pleurotus ostreatus were channelled on the determination of total polyphenols, total flavonoids and the percentage of inhibition of colour development (DPPH, Trolox equivalent), the latter being directly proportional to the antioxidant capacity of the extracts.

Determination of the total polyphenol content by the Folin-Ciocâlteu method

By the colorimetric reaction of oxidation-reduction determined by the Folin-Ciocâlteu reagent and calcium Carbonate, it was facilitated to determine the total polyphenols content of the four extracts obtained by different extraction methods and different solvents, respectively methanolic and ethanolic extracts of Pleurotus ostreatus, obtained under different ultrasonic and agitation conditions**.**

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The results of this quantification, calculated in relation to the standards of gallic acid on the standard curve, are given in Table 1.

Table 1. Determination of total polyphenols by Folin-Ciocâlteu method

Extraction method	*Polyphenols content (ug/ml) diluted 1:100*			*Sample weight (g)*	*Polyphenols content (ug/ml) undiluted*			*Polyphenols content (ug/g dw)*			*Total polyphenols content (ugGAE/g)*	
	Repl. 1	Repl. 2	Repl. 3	grame planta	Repl. 1	Repl. 2	Repl. 3	Repl. 1	Repl. 2	Repl. 3	**MEDIE**	**ST.DEV**
70% methanol + ultrasounds	0,0202	0,0206	0,0202	0,414	2,0166	2,0648	2,0199	14,6133	14,9625	14,6367	**14,74**	**0,20**
70% ethanol + ultrasounds	0,0199	0,0201	0,0201	0,415	1,9914	2,0131	2,0137	14,3953	14,5523	14,5566	**14,50**	**0,09**
70% methanol + stirring	0,0203	0,0203	0,0203	0,438	2,0333	2,0312	2,01257	13,9269	13,9269	13,8749	**13,90**	**0,03**
70% ethanol + stirring	0,0203	0,0202	0,0203	0,416	2,0278	2,0223	2,0329	14,6235	14,5840	14,6605	**14,62**	**0,04**

The three successive repetitions are shown in Figure 4, whilst Figure 5 indicates the results of the determination, respectively the average of the three repetitions in µgGAE/g dry matter.

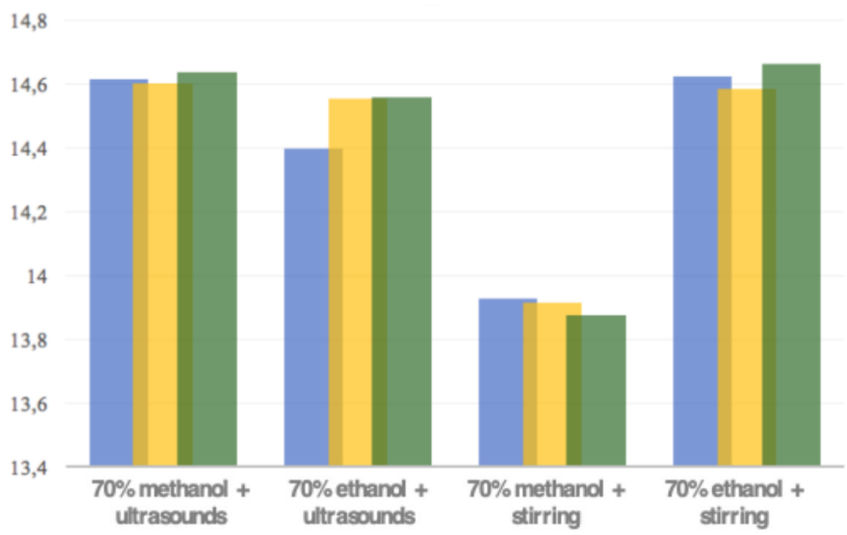

Figure 4. Total polyphenol values for the four extracts obtained from the three successive repetitions reported in mg / g dry matter

From the graphic representation (Figure 4) the highest value of total polyphenols was obtained for the methanolic extract of *Pleurotus ostreatus* subject to the ultrasonic procedure, respectively 14.74 ± 0.20 µgGAE / g. This was followed by the ethanol extract obtained by stirring (14.62 ± 0.04 µgGAE/g) and ultrasoning (14.50 ± 0.09 µgGAE/g), and the lowest value, 13.90/g was indicated by the methanol extract obtained through agitation.

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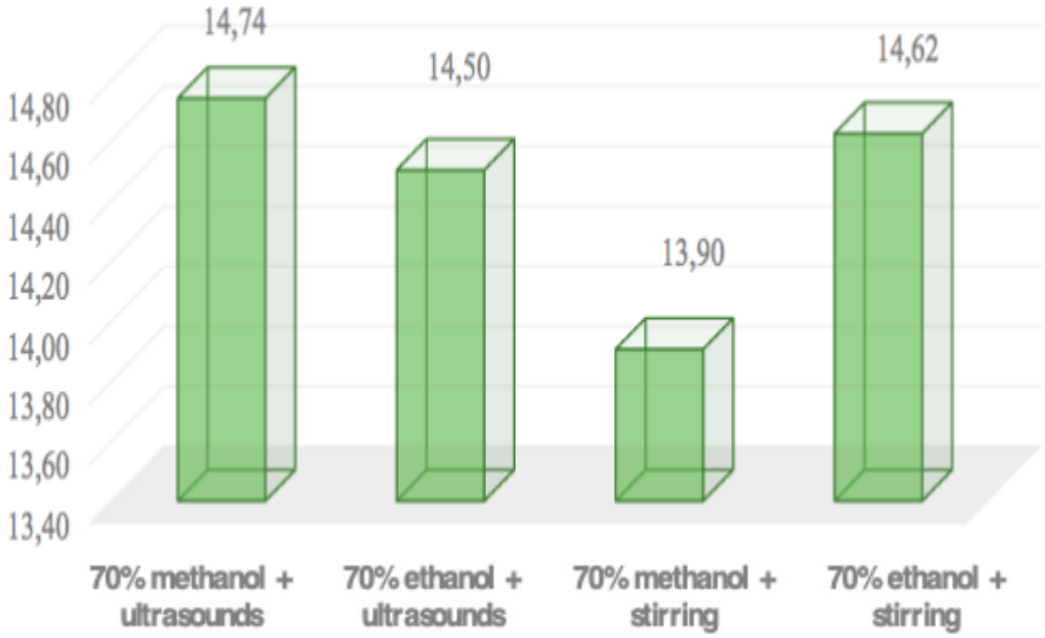

Figure 5. Total polyphenol content relative to the standard of gallic acid (µgGAE/g)

Determination of total flavonoid content by sodium nitrate method

The results presented in Table 2 indicate the total flavonoid content. Consecutive for reporting to the standard curve, performed after preparing solutions of known concentration from the routine standard, total flavonoids between 85.87 ± 0.09 µg / were obtained/ml and 305.04 ± 0.23 µg / ml (Figure 6). The minimum value was indicated by *Pleurotus ostreatus* extract in methanol obtained by ultrasonic, followed by the same methanol extract, but obtained by stirring with a value of 133.77 ± 0.57 µg/ml.

Table 2. Values in µg/ml for total flavonoid content

	Extraction methods	Calculated values			MU
1	MeOH ultrasonication	85,85	85,76	85,99	
		Average ± SD		***85,87 ± 0,09***	µg/ml
2	MeOH stirring	134,58	133,38	133,36	
		Average ± SD		***8133,77 ± 0,57***	µg/ml
3	EtOH ultrasonication	305,38	304,87	304,89	
		Media ± SD		***85,87 ± 0,09***	µg/ml
4	EtOH stirring	295,11	293,94	293,63	
		Media ± SD		***294,23 ± 0,63***	µg/ml

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At the upper pole, in addition to the ethanolic extract obtained by ultrasonic which indicated the largest number of total flavonoids (305.05 ± 0.23 µg /ml), the same ethanol extract is found but obtained by applying the stirring technique (294.23 ± 0.63 µg /ml).

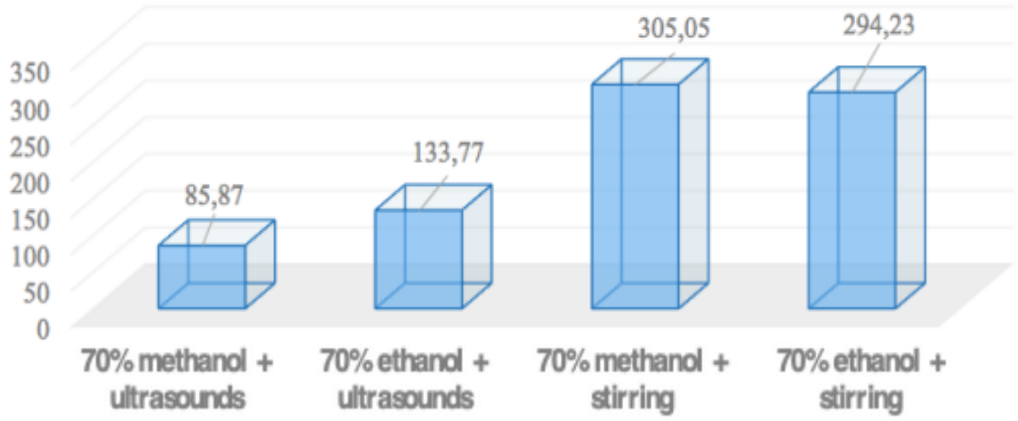

Figure 6. Total flavonoid content, relative to routine standard (µg/ml)

Evaluation of antioxidant activity by the DPPH method:

By using the 2,2-diphenyl-1-picrylhydrazil reagent, a quantification of the antioxidant activity of the four extracts was achieved. In the presence of antioxidants in their structure, the absorbance recorded for DPPH decreases, the decrease of these values being proportional to the antiradical activity. The more effective the compound, the greater the decrease.

The degree of discoloration of the DPPH reagent was calculated as an inhibition percentage, a percentage that reveals the antioxidant activity of the tested samples, the two being directly proportional. For the four alcoholic extracts subject to these determinations, the results are presented in Table 3.

Table 3. Antioxidant activity (% DPPH inhibition) for the four extracts

Antioxidant activity (inhibition % DPPH)			
Abs_{sample} **= 0,8003**			
Abs_{blank} **= 0,9881**			
$Abst_{rolox}$ **= 0,7814**			
$Conc_{trolox}$ **(µmol/ml) = 250**			
MeOH ultrasonication	**MeOH stirring**	**EtOH ultrasonication**	**EtOH stirring**
25,62 ± *0,72*	**29,692 ± *1,02***	**17,09 ± *0,29***	**18,78 ± *0,41***

The highest percentage of inhibition was recorded for the methanolic extract of Pleurotus o. Subject to the agitation technique, having a value of 29.69%. It was followed by methanol extract obtained by ultrasonic with a PI of 25.65%. For the other two ethanol extracts, the percentages did not exceed the value of 20%, respectively 18.78% for the ethanol extract obtained by stirring and 17.09% for the one obtained by ultrasoning (Figure 7).

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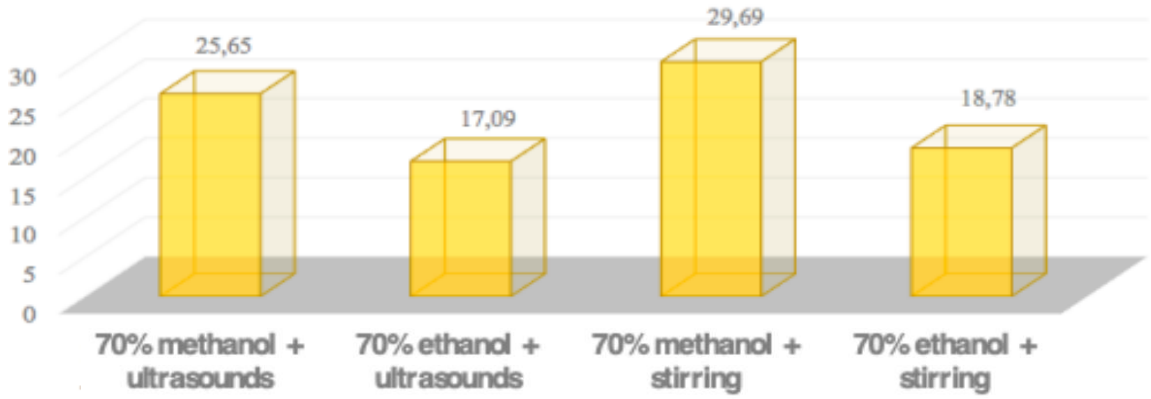

Figure 7. Inhibition rates (%) obtained by the DPPH method

To achieve an overview of the antioxidant determinations of the four Pleurotus o extracts., a double-axial graph was chosen to design the three methods of analysis applied (Figure 8), respectively total polyphenols, total flavonoids and the percentage of inhibition of DPPH directly correlated with the antioxidant capacity of the extracts.

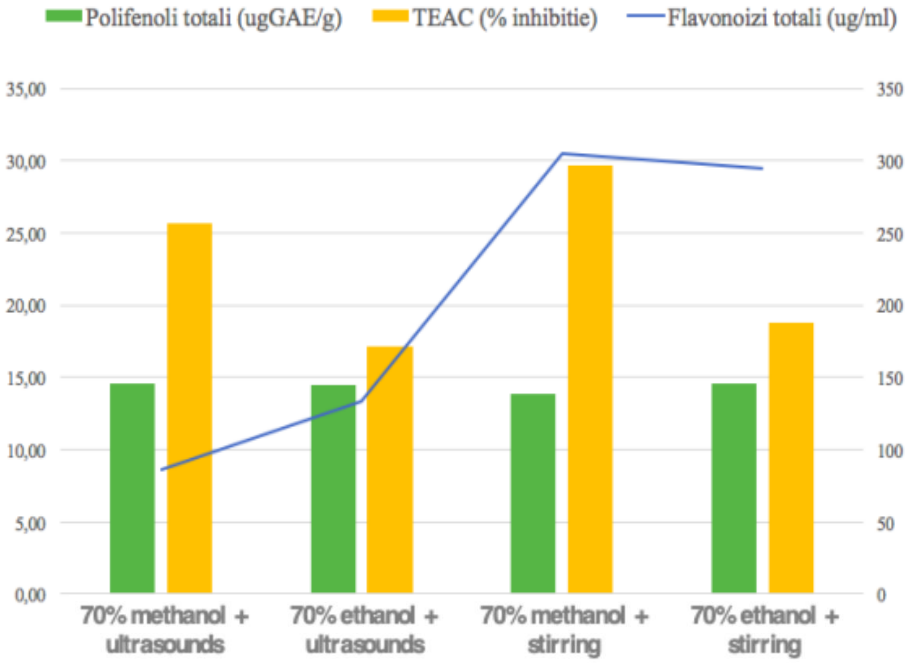

Figure 8. Double-axial graph with the three determinations applied to the extracts

Conclusions

The main objective of the study is to determine the antioxidant activity of beech sponges. Beech sponges are basidiomycete mushrooms characterized by their eccentric leg. They appear in October – February, from plain to mountain, on the rotten wood material of the deciduous trees. They are good for consumption; have significant amounts of protein, lipids and vitamins. They have a wide use of biotechnological interest.

The research was carried out in the Research Laboratory of antioxidant systems, A1c, within the complex of Research Laboratories "Horia Cernescu" of ULS, Timisoara related to the Research Institute for Biosafety and Bioengineers.

The determination of the antioxidant activity of beech sponges was performed by:

- determination of polyphenolic content (Folin-Ciocâlteu method).
- determination of total flavonoids (sodium nitrate method).
- evaluation of antioxidant activity by the DPPH method.

Results of determinations aimed at quantifying the antioxidant properties of *Pleurotus ostreatus* alcoholic extracts were channelled to determine total polyphenols, total flavonoids and the inhibition of colour development (DDPH, Trolox equivalent), the latter being directly proportional to the antioxidant capacity of the extracts.

The highest value of total polyphenols was obtained for the methanol extract of *Pleurotus ostreatus* subjected to the ultrasonic procedure, followed by the ethanolic extract obtained by agitation and ultrasonic, and the lowest value was indicated by the methanol extract obtained by agitation.

The minimum value of total flavonoids was indicated by *Pleurotus ostreatus* extract in methanol obtained by ultrasonic, followed by the same methanol extract, but obtained by agitation. At the upper pole, in addition to the ethanolic extract obtained by ultrasonic which indicated the largest number of total flavonoids, the same ethanol extract is found but obtained.

The highest percentage of inhibition of DPPH directly correlated with the antioxidant capacity of the extracts was recorded for the methanolic extract of *Pleurotus ostreatus* subjected to the stirring technique, followed by methanol extract obtained by ultrasonic, the ethanol extract obtained by agitation and that obtained by ultrasonic.

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