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ISOLATION, CHARACTERISTICS, AND ANTIOXIDANT ACTIVITY OF LOW MOLECULAR COMPOUNDS OF FRUIT BODIES LACTARIUS PERGAMENUS (Fr.) Fr MUSHROOMS

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Abstract

Methanol and methylene chloride extracts were obtained from fresh and dried fruit bodies of *Lactarius pergamenus* (Fr.) Fr. and studied by gas-chromatography–mass spectrometry (GC/MS), thin-layer chromatography (TLC), and column chromatography on silica gel. Antioxidant activity of individual fractions of mushroom extracts was measured by using DPPH (1,1-diphenyl-2-picrylhydrazyl). Stearic acid was removed from the extracts by their freezing, and phthalic acid esters, sesquiterpene compounds, long chain fatty acids, and their derivatives were found after that GC/MS analysis showed that the most abundant antioxidant compounds in methylene chloride extracts are: velleral dialdehyde, azulene sesquiterpenes, leaf and pelargonic aldehydes, and oleic acid amide. The velleral is known to provide a pungent taste of *Lactarius* sp. mushrooms. Thus, the antioxidant properties of methanol and methylene chloride extracts of mushrooms of *Lactarius* genus are not caused by the presence of phenols, flavonoids, β-carotenes, and tocopherols, as it was suggested in other studies. High level of phthalates that was discovered in these mushrooms, probably, is play an important role in the mechanisms of defence of mushrooms from insects. The obtained data are important for controlling chemical composition during isolation of pharmacologically active substances, and for preparation of antifungal ointments, in particular.

Keywords: *Lactarius pergamenus* (Fr.) Fr; TLC; GC/MS; low molecular compounds; antioxidant activity.

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1. Introduction

Semi-edible mushrooms of Lactarius genus are widely spread in moderate climate areas and are known to release a crushing milky sap containing substances with bitter, pungent or otherwise unpleasant taste. Compounds present in this sap were shown to possess antimicrobial, cytotoxic, antifeedant and even mutagenic properties [1].

Extracts of dried fruit bodies of L. pergamenus were used to prepare a composite which was effective for treatment of foot mycoses, as they suppressed growth of highly pathogenic fungus Candida albicans [2]. The trimethylfuranolactarane which was also purified from the above mentioned composite had a pronounced cytotoxic effect towards murine leukemia *L1210* cells [3]. It should be noted that a majority of components present in milky sap of mushrooms of Lactarius genus are very labile. The sap of some specimens changes its color when exposed to air (oxygen). This property is commonly used for identification of particular specimens of mushrooms belonging to this family. In addition, the taste characteristics of the mushrooms change after heat treatment. A fresh-cut peppery milk cap (L. pergamenus) has a “peppery” flavor of the milky sap which, however, quickly loses this flavor when exposed to air or after heat treatment. Meanwhile, a false saffron milk-cap (Lactarius deterrimus) is considered to be one of the most delicious agarics. Since some bitter after-taste still remains, such mushrooms are not very popular among the mushroom hunters. In some regions of the Ukrainian part of the Carpathian mountains, the local common name of L. pergamenus mushroom is close to “bitterness”. Interestingly, not only drying but also freezing of fruit bodies of L. pergamenus result in the loss of mushroom peppery flavor.

The above mentioned properties of fruit bodies of Lactarius genus mushrooms suggested that there exist some labile substances responsible for the burning taste. Two isomeric substances were purified from Lactarius vellereus fruit bodies in 1969 after numerous unsuccessful attempts. These were velleral and isovelleral that caused the “peppery” taste of the fresh-cut fruit bodies [4]. A few years later, these compounds were also isolated from L. pergamenus fruit bodies [5]. It was also shown that methanol or ethanol extraction, especially while heating, led to the formation of artifacts of furanolactarane structure, without the peppery taste that is characteristic of the fresh mushrooms [6]. We found that 3,14,15-trimethylfuranolactarane-8-ol purified by methanol extraction and subsequently by chromatography on Silica gel retained sufficiently both high biological activity and relatively stable during its long-term storage without air admission [3]. Other substances present in fractions isolated by the Silica gel column chromatography of methanol extract from dried L. pergamenus fruit bodies, also possessed some biological activity [2,3] that might be related to their antioxidant properties.

It is known that the milky sap of mushrooms is rich in lipids and lipophilic compounds, while free radicals can initiate lipid peroxidation. Lipids stabilize the emulsion, and their oxidation might cause destabilization of the milky sap of mushrooms of Lactarius genus. In was demonstrated that the antioxidant properties of fruit bodies of closely related mushroom Lactarius piperatus depended on the stage of their maturity [7]. Similarly, chemical composition and antimicrobial activity of substances present in the fruit bodies also depended on the maturity of mushrooms [8]. Usually, more mature fruit bodies showed lower antimicrobial and antioxidant activity and a decreased level of saturated fatty acids, while the content of unsaturated fatty acids was increased [8]. The authors of those studies [7,8] relate such dependence with changes in the ratio of total phenols, flavonoids, ascorbic acid, beta-carotene and lycopene.

Here we present a detailed investigation of the chemical composition of dried fruit bodies of Lactarius pergamenus (Fr.) Fr as an attempt to clear up the nature of antioxidant substances in this mushroom. We also address the dependence of the composition upon drying procedure, and a possibility of artifacts introduced by the analytical methods using specific chemical reactions and TLC. The obtained data are important for controlling chemical composition during isolation of pharmacologically active substances, and for preparation of antifungal ointments, in particular.

2. Materials and Methods

2.1. Samples and samples extraction

Fruit bodies of L. pergamenus were collected in July-August in the area of abundant appearance in mixed coniferous – deciduous forests near Skole of Lviv region (Ukrainian part of the Carpathian mountains). Mushrooms were delivered to laboratory within 12 h of collection. Some of the mushrooms were placed in a desiccator at +52°C for 24 -48 h, and the remaining fresh mushrooms were ground in a meat chopper and processed to a sap by pressing (and squeezed to obtain sap). The sap was used for the isolation of a lectin [9], while the remaining pomace was dried out in a desiccator at +52^{o}C and used for further extraction and investigation.

After drying, the fruit bodies and pomace were ground to powder (D<0.5 mm), placed in the Soxhlet extractor and extracted for 3-6 h by: a) methanol; b) methylene chloride; c) petroleum ether. The majority of solvent was sublimated, and its residue was evaporated in a desiccator at +52°C.

Extraction of the lipophilic substances from fresh mushrooms was carried out using various solvents at different temperatures. A batch of fresh fruit bodies was flooded with methanol (1:4), and vigorously mixed at room temperature for 5 min by the electric blender. The water-methanol homogenate was further squeezed out through dense fabric, and centrifuged at 6 000 g for 10 min. The supernatant was extracted twice with equal volume of methylene chloride, and the extract was filtered through the anhydrous sodium sulfate. Solvent was evaporated, and the residue was weighed and used for further analyses.

Alternatively, a batch of fresh fruit bodies was homogenized with water (1:4), and methylene chloride was added in quantity equal to half of the volume of the homogenate. Mixture was agitated by the mechanical shaker for 30 min, after which it was centrifuged. The lower organic layer was collected for furher experiments.

One gram of dry residue obtained from the methanol extract of dried fruit bodies, was further extracted with the following solvents: hexane (20 ml) → chloroform (15 ml) → diethyl ether (12 ml) → ethyl acetate (12 ml) → butanol (12 ml) → isopropanol (10 ml) → methanol (10 ml) → water (15 ml). The obtained extracts were marked as fractions 1 - 8, according to the extraction procedure, and used to study their chemical composition and antioxidant activity.

2.2. Samples chromatography

The extraction of fresh and dried fruit bodies with methylene chloride was advantageous compared to the extraction with hexane or petroleum ether (see "Results and Discussion"), and thus, that extract was subjected to more thorough investigation. The residue of extract obtained by methylene chloride (2.8 g of dark-brown solid mass) was dissolved in 12 ml of methylene chloride (high A.R. quality) and then applied onto Silica gel L 40/160 column (40 x 2 cm) that was preconditioned by methylene chloride. After that, the column was subsequently eluted with the following solvents: methylene chloride (200 ml), methylene chloride - ethyl acetate at 1:2 ratio (300 ml), methylene chloride - ethyl acetate – methanol at 4:2:1 ratio (300 ml), and, finally, with methanol (200 ml). At this point the column was completely cleared of pigmented substances. Based on absorption measurements of the eluate at 450 nm, six fractions were formed and designated as fractions 1.1 - 1.6. The TLC analysis of these fractions on the Silufol plates (Kavalier, Czech Republic) showed that they contained a complex mixture of substances.

The fractions obtained after column chromatography were investigated by gas chromatography – mass spectrometry (GC-MS) using 6C/MS Agilent Technologies 6890 N/5975 B instrument. One microliter injections were applied to the HP-5 column (30 m length and 0.25 mm ID), filled with of 5% phenyl, 95% dimethylpolysiloxane stationary phase. Helium was used as gas carrier at 1.5 ml/min flow rate. The column was washed with methanol. The GC oven program was isothermal at 75^{o}C, then a ramp at 15^{o}C /min to 300^{o}C, then again isothermal at 300^{o}C for 8 min. The mass-selective detector interface temperature was set at 250^{o}C. The ion source was operated by electron ionization, the ionizing energy was 70 eV, ion source temperature 230^{o}C, quadrupole temperature 150^{o}C. The mass spectrometer was operated in scan mode. The identification of compounds in samples was performed using the GC-MS libraries.

Additionally, the detection of substances present in each fraction, was monitored by TLC on the Silufol plates. Chromatography was conducted using n-hexane - ethyl-acetate (8:1) and n-hexane - ethyl-acetate - methanol (4: 2: 1) eluents, which provided the best separation. After air drying, TLC plates were examined under the UV light, developed with one of the following: 0.01% DPPH (1,1-diphenyl-2-picrylhydrazyl free radical) solution in acetone, 0.3 % o-dianisidine solution in methylene chloride, 0.05 % water-acetone solution of potassium permanganate, iodine vapor, concentrated sulfuric acid, solution of 1 % vanillin in concentrated sulfuric acid, and saturated solution of phosphorus-molybdenum acid in methanol.

2.3. Antioxidant activity

Antioxidant activity of low molecular weight substances present in the extract, was measured by a reaction with DPPH which is used for colorimetric studies of redox processes (Asatiani et al. 2007; Barros et al. 2007a). DPPH is dark violet, and its reduced derivative is orange-yellow. DPPH was prepared as a 0.5 mM solution in methylene chloride. In order to estimate total antioxidant activity, 2.0 ml of 0.5 mM solution of DPPH in methylene chloride were added to 0.5 ml of solution of individual fractions or substances isolated from mushroom material and taken in three different concentrations (in the range of 1-10 mg/ml). The solvent was used as control. After 15 min mixing, the solution absorbance was measured by spectrophotometer at 520 nm. The results are expressed as a percentage of reduction of the initial DPPH radical absorption by the test samples. Solutions of ascorbic acid, rutin and amide of oleic acid were used as positive controls for measuring antioxidant activity. Percent inhibition of DPPH free radical (I%) was calculated as follows: I% = (Ablank-Asample) / (Ablank)×100, where Ablank is the absorbance of control reaction (containing all reagents except the test compound), and Asample is the absorbance of the test compound. Concentration of the extract providing 50% inhibition (EC50), was calculated from the linear regression of the plot of inhibition percentage versus the concentration of the extract. All analyses were performed in triplicate.

Detection of the phenolic compounds in methanol or water-methanol extracts or solutions was conducted by the reaction with iron (III) chloride, and flavonoids - by the reaction with strong bases, Bryant's cyanidin reaction and classic Wilson's citrate-boric reaction, as described in [10] .

All chemical reagents used in the study were of analytical grade or higher. Oleic acid amide was synthesized according to the method described previously [11].

2.4. Statistical analysis

All determinations were done in triplicate. Standard deviation was calculated, and statistical significance of the differences was determined using Student's t-test ($P<0.05$).

3. Results and Discussion

Using fresh mushroom fruit bodies for isolation of biologically active substances may be inconvenient, since it requires immediate processing. Usually, freezing does not affect biological activity of natural substances. However, in case of L. pergamenus fruit bodies, the hemagglutination titer of specific lectin decreased 4-8 times after freezing [9], and the burning taste of mushrooms practically disappeared [4]. Dried mushrooms are much easier for use. Drying at +52^{o}C of 1.0 kg of fresh cleaned of soil L. pergamenus fruit bodies yields 95±15 g of dried mushrooms.

ISSN 2347-6893

3.1. Extract examination by TLC chromatography

Extraction of dried L. pergamenus fruit bodies with organic solvents led to a considerably (3 - 7 times) higher yield of the end product relative to extraction from fresh fruit bodies. However, distinct differences between extracts isolated from fresh and from dried mushrooms were detected by the TLC analysis. Examination under UV light of the chromatograms of extracts of dried mushrooms, revealed a spot with intensive yellow-green fluorescence, which was absent on chromatograms of extracts of fresh mushrooms. In addition, the examination under UV light of the chromatograms of fresh mushroom extracts, showed a dark spot appearing after 30–90 min, whose intensivity strongly encreased, in 12 – 24 h. Such phenomena were not observed on the chromatograms of extracts of dried mushrooms (Fig.1).

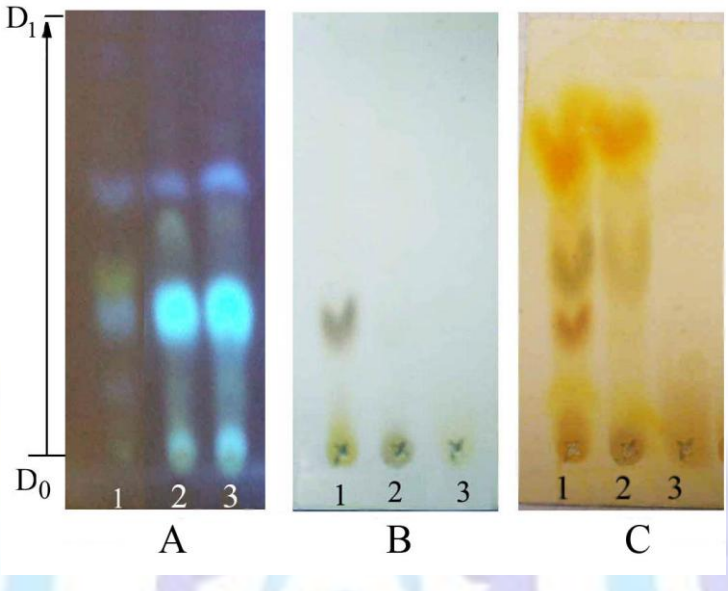

Fig. 1. TLC on Silufol plates of the extracts isolated from *L. pergamenus* fruit bodies

Hexane : ethyl acetate (8:1) solvent system. **D_0** – start line, **D_1** – solvent front

A - TLC plate under UV light;

B - same TLC plate under visible light 22 h after chromatography;

C - TLC plate after treatment with 0.3 % o-dianisidine solution in methylene chloride.

1 – fraction extracted from fresh mushrooms with methylene chloride;

2 – fraction extracted from fresh mushrooms with methanol and further extraction with the petroleum ether;

3 – fraction extracted from dried mushroom pomace with methylene chloride.

Therefore, drying at high temperatures (+50^o - 60^o C) led to formation of substances that are absent in the tissues of fresh mushrooms. This observation was also supported by the loss after drying of the acrid and pungent taste characteristic for fresh mushrooms. Evidently, substances with such properties can be obtained only from fresh mushrooms. However, their isolation is accompanied by considerable methodological difficulties.

The highest quantity of substances extracted from dried L. pergamenus fruit bodies, were obtained using methanol as solvent (Table 1). Extraction of the homogenate of fresh fruit bodies with methanol, which was then removed by distillation and further extraction of water concentrate with petroleum ether resulted in approximately six times lower yield of the end product relative to the same treatment of dried fruit bodies (1.26 % and 7.3 %, respectively; amounts of fresh mushrooms were recalculated to dry fruit bodies). It should be noted that dry residue of such extract did not possess a burning taste. Direct extraction of fresh fruit bodies with petroleum ether resulted in a lower yield of the end product comparing to the yield when the methylene chloride extraction was performed (2.32 % and 3.60 %, respectively, calculated per mass of dried material. Dry residues of those extracts possessed a noticeable burning taste.

Low yield of substances extracted by the organic solvents from water homogenates of fresh mushrooms, is probably due to loss caused by the separation of the mixture organic solvent - water homogenate of fruit bodies. Formed stable emulsions cannot be effectively separated even by a prolonged centrifugation (6 000 g, 30 min). Loss of the petroleum ether (due to emulsion formation) from the 1:3 mixture petroleum ether / water homogenate of fruit bodies reached 50-70 %, while the loss of the methylene chloride upon the separation of similar mixture was much less - 20 - 30 %. Therefore, further study of fresh mushrooms was carried out without extraction by petroleum ether. The main advantage of using methylene chloride for extraction of easily oxidized substances, is attributable to its low boiling temperature (+40^{o}C) that allows better protection of the thermolabile compounds (Table 1).

ISSN 2347-6893

Table 1.

Effects of extracting solvents on the yield of extractive substances from *L. pergamenus* fruit bodies and their antioxidant activity (EC_{50}^{DPPH}).

№	Raw material	Extracting solvent	Yield of extractive matters, %	EC_{50}^{DPPH}, mg/ml
1	Dried fruit bodies	Methanol + further extraction (Petroleum ether)	35.43 7.3	3.0
2	Dried pomace	Methanol + further extraction (Petroleum ether)	15.78 8.18	6.25
3	Fresh fruit bodies	Methanol + further extraction (Petroleum ether)	0.12 (1.26 *)	32
4	Fresh fruit bodies	Methylene chloride	0.34 ±0.3 (3.6 *)	27
5	Dried pomace	Methylene chloride	9.2	32
6	Fresh fruit bodies	Petroleum ether	0,22 (2.32 *)	16
7	Dried pomace	Petroleum ether	6.45	32

* - in recalculation to the air dried raw material.

The DPPH assay showed relatively low antioxidant activity of the total extracts of fresh and dried mushrooms (Table 1). One reason for that might be disintegration of sesquiterpene esters with dissociation of stearic acid, which stabilizes these compounds in the uninjured fruit bodies. However, when the mushrooms are injured, the sesquiterpene esters were rapidly reduced in the enzymatic reactions with uncoupling of both stearic acid and sesquiterpenes which are unstable on air [12,13].

Freezing of methanol extracts at -18oC produces the sediment consisting of almost pure stearic acid, with a yield of about 30 - 36 % (w/w) of the initial extract. The same procedure performed with petroleum ether and methylene chloride, gives extracts with higher yield of stearic acid reaching 50-70 % (Table 2).

Table 2

Content of stearic acid in the extracts isolated from *L. pergamenus* fruit bodies by using various extracting solvents

№	Raw material	Extracting solvent	Stearic acid, %
1	Dried fruit bodies	Methanol	34±5
2	Dried pomace	Methanol	37±5
3	Fresh fruit bodies	Methanol + further extraction (Petroleum ether)	69.9
4	Fresh fruit bodies	Methylene chloride	54.8
5	Dried pomace	Methylene chloride	59
6	Fresh fruit bodies	Petroleum ether	41.4
7	Dried pomace	Petroleum ether	62.5

Development of chromatograms with DPPH solution in acetone over a few minutes revealed yellow spots of easily oxidized substances on a blue background. The spots of fractions of fresh fruit bodies become visible in 3 -10 min, while the appearance of such spots of dried fruit bodies fractions takes much longer time - 30-60 min and more. The development of chromatograms with solutions of potassium permanganate and o-dianisidine revealed evidently the same substances. A use of 0.3% o-dianisidine solution in methylene chloride has certain advantages, since those chromatograms were stable during few days. The revealed spots had well defined border and were of distinct color which can help to identify their chemical nature. Meanwhile, the spots of substances detected with potassium permanganate solution, were unstable, poorly defined, and disappeared within one hour (Fig. 2). Besides, potassium permanganate detects oxidized substances much easier comparing with o-dianisidine solution.

When the TLC with the extracts from fresh mushrooms were used, the substances with Rf = 0.23 can be detected in a few hours after chromatogram exposed to air as a black spot (Fig. 1-B-1, Fig. 2-D-3), while they were absent in the extract from frozen mushrooms. Instead of that, light brown spot with the same Rf value could be seen in extracts of frozen mushroom (-18^{o}C) in 4–12 hr after exposure to air. Meanwhile, there are no changes in quality and quantity of spots after the TLC of the extract of dried mushrooms (Fig. 2-D-2). Evidently, that substances of fresh, frozen and dried fruit bodies differ in chemical composition.

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When the extract from fresh mushroom fruit bodies extracted with methylene chloride was stored in refrigerator (-18^{o}C) in a densely closed container that had no air, the substance of this extract with Rf = 0.23 appeared as the black spot even when it was stored for a protracted time.

In order to investigate chemical composition of substances extracted with methanol from dried mushrooms, which are responsible for changes in the chromatograms, as noted above, that extract was separated into 8 fractions by a successive extraction with various organic solvents. All these fractions were further analyzed by GC-MS. Also, their total antioxidant activity was measured with DPPH assay. The results of these studies are presented in Table 3.

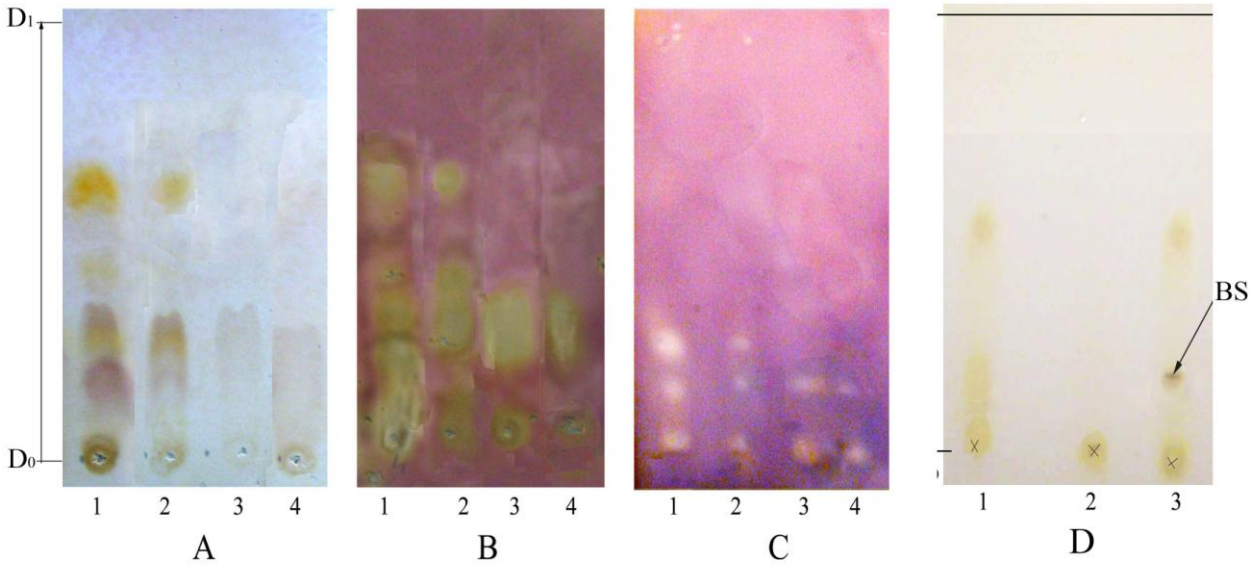

Fig. 2. TLC on Silufol plates of the extracts isolated from *L. pergamenus* fruit bodies

Hexane : ethyl acetate (8:1) solvent system. **D_0** – start line, **D_1** – solvent front.

A – TLC plate 22 hr after developing with 0.3 % o-dianisidine solution in methylene chloride,

B – TLC plate 10 min after spraying with 0.05 % potassium permanganate solution in acetone

C - TLC plate 30 min later after developing with 0.01 % DPPH solution in acetone.

1 – Fraction extracted with methylene chloride from fresh fruit bodies mushrooms;

2 – Fraction extracted with petroleum ether from fresh fruit bodies mushrooms;

3 – Fraction extracted with petroleum ether from dried fruit bodies mushrooms;

4 – Fraction extracted with methylene chloride from dried mushroom pomace.

D - TLC on Silufol plates of methylene chloride fraction without development and after 36 h of ambient exposure:

1 – frozen fruit bodies mushrooms;

2 – dried fruit bodies mushrooms;

3 – fresh fruit bodies mushrooms.

BS – a black spot

As can be seen by viewing the fourth column of Table 3, the bulk of easily oxidized substances of the methanol extract of dried mushrooms is present in fractions - 1, 2 and 7. Among them, fraction 1 is of most interest since it contains a large number of substances with antifungal and cytostatic activity [2,3]. Therefore, further fractionation of fraction 1 was carried out by chromatography on the Silica gel column (see “Materials and Methods”).

Fraction 7 dissolved in 70 % water methanol did not react chemically with alkali solution, gave a negative result of Bryant's cyanidin reaction, Wilson’s citrate-boric reaction, and with iron (III) chloride. Therefore, it was concluded that flavonoids and phenolic compounds are absent in this fraction.

ISSN 2347-6893

Table 3

Qualitative and quantitative composition of fractions obtained from the methanol extract of dried *L. pergamenus* fruit bodies, and their antioxidant activity (EC_{50}^{DPPH})

Fraction number	Extracting solvent	Yield, %	Chemical composition of the obtained fractions (identification by MS) and their content (%)	EC_{50}^{DPPH}, mg/ml
1	Hexane	24.7	More than 60 identified substances including sesquiterpenes	7.1
2	Chloroform	5.55	1. Palmitinic acid – 1.79 2. Oleic acid – 2.83 3. Stearic acid – 85.8 4. *Humulane-1,6-dien-3-ol – 2.15* 5. *Lasiocarpenonol – 3.24* 6. *Octahydro -7-methyl-benzacridine – 4.19*	13.3
3	Diethyl ether	1.65	More than 30 unidentified compounds	–
4	Ethyl acetate	1.0	1. Glycerol – 6.3 2. Palmitinic acid – 1.03 3. Stearic acid – 87.54 4. *N,N'-dimethyl-propanamide – 3.87* 5. *8-Damascone – 1.26*	–
5	Butanol	3.0	1. Glycerol – 21.51 2. Glycerol triacetate – 14.12 3. Glycerol α-monoacetate – 3.34 4. Stearic acid – 61	–
6	Isopropanol	2.0	1. Glycerol – 40.2 2. Stearic acid – 59	–
7	Methanol	9.5	More than 30 unidentified compounds	9.2
8	Distilled water	48.35	1. Mannitol 85.3 2. Sodium stearate 14.74	–
	Insoluble residue	0.35	Analysis was not performed	–
	Total	96.1		

Note: Italic font is used in the Table to denote substances with less than 90 % reliability of GC-MS results. The absence of decrease in absorbance of the reaction mixture after 15 min at 10 mg/ml concentration of the substance in a solution is marked by dash.

The Silica gel column elution profile of the methylene chloride extracts obtained from both fresh and dried mushrooms is presented in Fig. 3. Six fractions by measuring the absorbance at 450 nm were obtained. The presented graphs demonstrate only small quantitative differences between the extracts that were obtained from fresh and from dried mushrooms.

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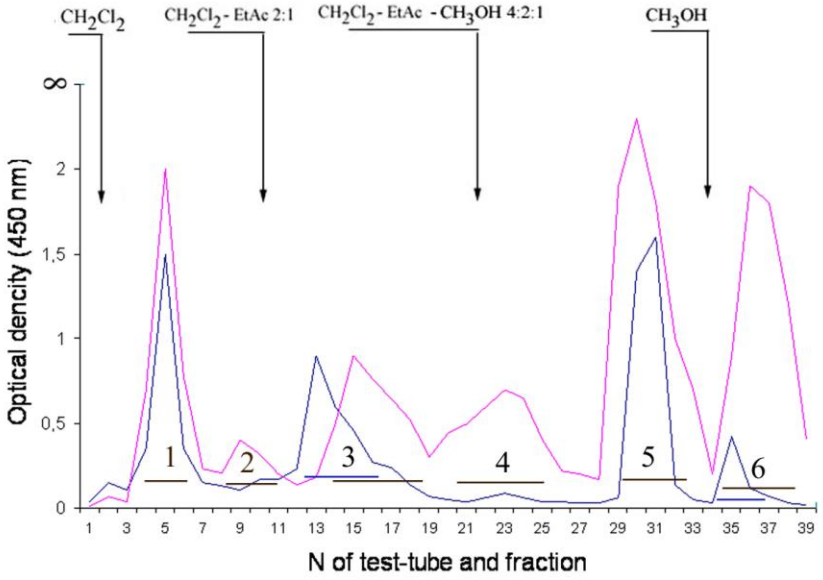

Fig. 3. Elution graph from the Silica gel column of the methylene chloride extracts obtained from fresh (blue line) and dried (pink line) L. pergamenus fruit bodies.

The extinction values at 450 nm do not reflect true quantitative relationships between the fractions obtained from fresh and dried mushrooms. Because of that, for quantitative analysis each fraction was dried and weighed. The results are presented in Fig. 4A.

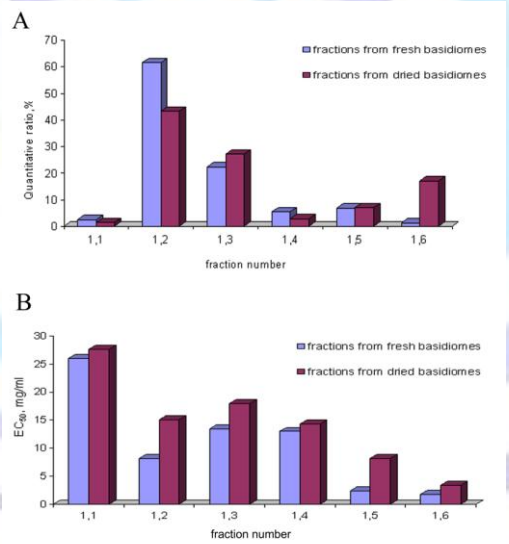

Fig. 4. Ratio of fractions (A) and qualitative and quantitative correlation of the antioxidant activities of fractions (B) of the methylene chloride extracts obtained from fresh and dried L. pergamenus fruit bodies.

The diagram in Fig. 4A shows that in both fresh and dried fruit bodies fraction 1.2 predominates quantitatively, while the amount of fraction 6 is higher in the extracts from dried fruit bodies comparing to the extracts of fresh fruit bodies.

3.2. Analysis of antioxidant activities

Analysis of antioxidant activities (evaluated by using DPPH assay) of the fractions obtained from fresh and dried mushrooms is presented in Fig. 4B. The antioxidant activity is inversely proportional to the numeric coefficients, i.e. substances with low coefficients have high antioxidant activities. These data show that fractions isolated from fresh mushrooms have higher antioxidant activity than the corresponding fractions isolated from dried mushrooms. Besides, the fractions eluted from the column later possess higher antioxidant activity than the early eluted fractions. It should be noted that in comparison with the activity of well known antioxidants, the antioxidant activity of individual fractions of L. pergamenus is insignificant, since the EC_{50}^{DPPH} values for rutin and ascorbic acid were 0.04 mg/ml and 0.038, accordingly.

Qualitative and quantitative composition of the obtained fractions analyzed by the GC-MS, are presented in Table 4.

ISSN 2347-6893

The analysis of these data shows that the methylene chloride extracts from both fresh and dried L. pergamenus fruit bodies are characterized by high content of phthalic acid esters, sesquiterpenes (mainly azulenes), long chain fatty acids, and their derivates (mainly stearic and oleic acids) and relatively small quantities of other substances. An interesting finding is that fractions 1.2 and 1.3 isolated from dried fruit bodies are characterized by high content of the oleic acid. These results are in agreement with the earlier conclusion that the content of the unsaturated fatty acids increases as the mushrooms age [8]. Mushroom drying resulted in a decrease of saturated hydrocarbon content in the methylene chloride extract from fresh mushrooms, and in an increase of oleic acid amide content in the similar extract obtained from dried mushrooms.

The phthalic acid esters were visualized by exposing the chromatograms to the iodine vapor. They appear as white spots on a violet background, and in 8-16 h they become brown on colorless background (Fig. 5). Phthalates do not reduce DPPH, however they react with potassium permanganate which results in the appearance of yellowish-brown spots on a pink background. This indicates that phthalates are not easily oxidized in open air, and that they are present in large amount in fresh and dried fruit bodies.

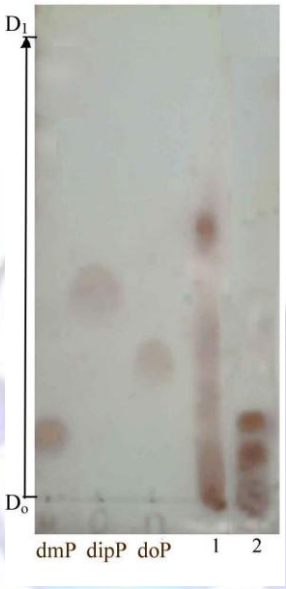

Fig. 5. TLC on Silufol plates of pure phthalates and fractions 1.1 and 1.2 obtained from the methylene chloride extract of dried L. pergamenus fruit bodies after exposuse to iodine vapor and 18 h ambient exposure.

Hexane : ethyl acetate (8:1) solvent system. **D_0** – start line, **D_1** – solvent front.

1 – fraction 1.1;

2 – fraction 1.2;

dmP – dimethylphtalate;

dipP – diisopropyl phthalate;

doP– dioctyl phtalate.

During development of TLC plates with 0.05 % $KMnO_4$, it was noticed that oleic acid appears as a yellow spot on a pink background, in contrast to stearic acid which is not visible. Both of these acids can be detected as brown and white spots, accordingly, when exposed to the iodine vapor. DPPH and o-dianisidine are not capable of visualizing these substances. Sulfuric acid which is a universal visualization reagent for various organic compounds, revealed (without heating the chromatogram) oleic acid as a pink spot, while the stearic acid did not appear.

Phosphorus-molybdenum acid, potassium permanganate and iodine vapor are used in TLC as developers to detect antioxidants. However, these reagents are not as specific [14,15]. Potassium permanganate and iodine vapor are used to visualize unsaturated compounds, while terpenoids, diterpenoid acids, sesquiterpene alcohols, sesquiterpene lactones, azulenes, higher alcohols, ketones are detected using a reaction with vanillin in concentrated sulfuric acid and phosphorus-molybdenum acid.

One can see from Fig. 6, that phosphorus-molybdenum acid can be used to visualize under UV light almost all substances in this study.

As shown in Fig. 6, the highest antioxidant activity, as measured using the DPPH assay, was characteristic of fraction 1.6 of the methylene chloride extract obtained from both fresh and dried fruit bodies and fractionated on the Silica gel column. According to the GC-MS data, fraction 1.6 isolated from dried fruit bodies contains high amount of the oleic acid amide. This substance possesses moderate antioxidant activity (EC_{50}^{DPPH} = 2.0 mg/ml). It should be noted that storage on the open air of the individual fractions obtained even from dried fruit bodies extracts, causes a decrease in their

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antioxidant activity. For example, after storage for 24 h on the open air, fraction 1.6 possesses EC_{50}^{DPPH} = 3.5 mg/ml, and after storage for 44 days its EC_{50}^{DPPH} = 8.4 mg/ml. However, the content of this amide is relatively low in the same extract obtained from fresh mushrooms.

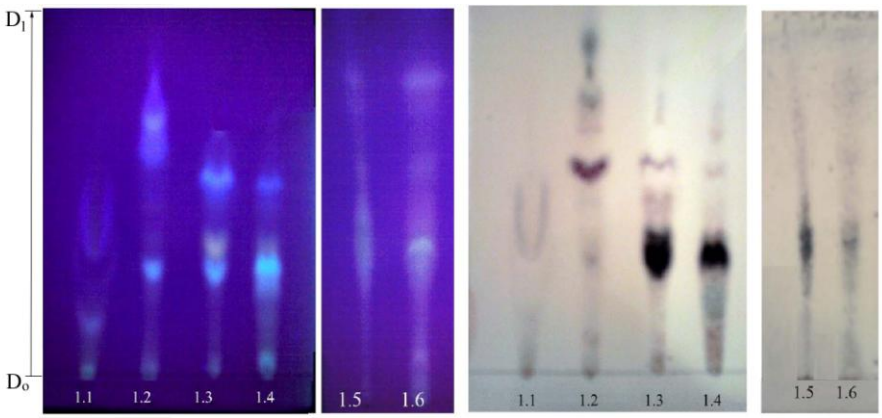

Fig. 6. TLC on Silufol plates of fractions 1.1 - 1.6 obtained from the methylene chloride extract of fresh L. pergamenus fruit bodies . UV light detection is on the left, and treatment with phosphorus-molybdenum acid is on the right.

Hexane : ethyl acetate (8:1) solvent system was used for fractions 1.1 - 1.4, and hexane : ethyl-acetate : methanol (4:2:1) solvent system – for fractions 1.5 - 1.6. D0 – start line, D1 – solvent front.

In some studies [7, 16,17,18], it was suggested that the antioxidant properties of higher mushrooms, in particular of Lactarius genus, are caused by the presence of phenols, flavonoids, β-carotenes, and tocopherols. Hovewer, the results of our investigation based on using TLC and GC-MS, have shown that these substances possessing antioxidant properties are absent in the methylene chloride extracts of L. pergamenus fruit bodies. According to our data, the antioxidant activity of this semi-edible mushroom is caused by the presence of the velleral dialdehyde, azulene sesquiterpenes, leaf and pelargonic aldehydes, and amide of the oleic acid.

High level of phthalates was discovered in the fruit bodies of mushrooms. Phthalates play an important role in the mechanisms of defence of mushrooms from insects. High level of phthalates in these mushrooms, probably, is necessary for fungus body for protection from attacks by insects. It is known that dimethyl or diethyl phthalates are used in veterinary medicine as insect repellent in some topical formulations. Dimethyl phthalate is an antiparasitic drug (ectoparasiticide), used in the treatment of ectoparasitic infestations such as nematodes, cestodes, trematodes, infectious protozoa, and amoebas. It can be applied for treatment of rickettsial infections, as a miticidal agent (Karunamoorthi , Sabesan, 2010). Tajegny-antimosquit creams, emulsions, lotions for protection from mosquito are widely used in Russia (Siberia). Thus, the presence of phthalates in living organisms is not frequent, but it occur, for example, in plants, for example in Helichrisum arenarium and perhaps, are characteristic for mushrooms of Lactarius genus.

Table 4. Qualitative and quantitative composition of the fresh and dried fractions *L. pergamenus* fruit bodies

Fraction number	Chemical composition of the obtained fractions (identification by MS, main components)					
	Fresh mushrooms			Dried mushrooms		
	Name of substances	Content (%)	Degree of precision	Name of substances	Content (%)	Degree of precision
1	**2**	**3**	**4**	**5**	**6**	**7**
1.1	Diisooctyl phthalate	50.15	91	Diisooctyl phthalate	54.43	91
	Phthalic acid butyl cyclohexyl ester	7.65	90	Butyl-2-ethylhexyl phtalate	2.24	83
	Dibutyl sebacinate	8.17	87	Eudesma-5,11(13)-dien-8,12-olide	17.75	55
	Saturated hydrocarbons:			9-cis-Oleic acid	12.13	99
	- Octadecane	5.69	98	Ethyl Oleate	4.91	99
	- Tetradecane	3.00	97	Di-(9-octadecenoyl)-glycerol	2.01	38
	- Octacosane	3.38	91			
	- Eicosane	3.20	25			

	Methylnaphthalene	2.19	91			
	Velleral	3.98	47			
	Gibberellin A_3	2.04	43			
	Other 3 substances	10.45		*Other 17 substances*	6.53	
1.2	Stearic acid	58.17	99	Diisooctyl phthalate	25.93	91
	(4aS,7R,8aS)-6,9,9-Trimethyl-4,4a, 7,8,8a,9-hexahydronaphtho[2,3-b]furan-7-ol	16.00	90	9-Octadecenoic (oleic) acid	22.86	99
	Eudesma-5,11(13)-dien-8,12-olide	10.56	47	Glycerol 1-monooleate	8.71	64
	Bis(2-ethylhexyl) phthalate	5.80	78	Decanedioic acid dibutyl ester	3.57	90
	1-Indanone	4.94	64	Caprynic acid	1.75	97
	Costunlide	2.19	60	Palmitinic acid	1.52	99
	Dibutyl phthalate	1.47	83	2-Naphthaleneacetaldehyde	1.16	53
				Ergosterin	1.58	99
				1-methyl-6,7-dimethoxy-8-formamido isoquinoline	1.17	80
				gamma-Sitosterol	1.08	99
	Other 2 substances	0.87		*Other 55 substances*	30.67	
1	**2**	**3**	**4**	**5**	**6**	**7**
1.3	cis-1-((Phenylthio)methyl)-2-(2-propenyl) cyclopentane	34.62	38	Azuleno[5,6-c]furan-1(3H)-one, 3a, 4,4a,5,6, 7,7a,8-octahydro-4-hydroxy-6, 6,8-trimethyl	15.36	97
	Isocritonilide	20.27	90	Critonilide	13.50	90
	Stearic acid	5.02	99	Isocritonilide	6.87	83
	2-methyl-3-(m-trifluromethylphenyl)-2-butene	4.90	49	Oleic acid	10.56	99
	2-Methylthio-3,4-dihydrobenzooxepino [2,1-c]thiophene	4.52	83	Dibutyl sebacate	6.32	90
	l-methoxy-3,4,5,7-tetramethylnaphthalene	3.48	72	5-Cycloocten-l-one	3.51	55
	lH-Indole-2-methanamine	2.71	72	alpha-Gurjunene	2.20	78
	Dibutyl phthalate	1.72	59	Dibutyl phthalate	1.93	46
	2,2, 4-Trimethylfuro[6, 7-c]-1, 3,8H-azulene	1.61	90	Velleral	1.56	60
	Other 4 substances	21.15		*Other 26 substances*	38.19	
1.4	alpha.-Gurjunene	13.14	87	Isocritonilide	23.35	90
	Isocritonilide	9.42	90	Clovene	12.01	41
	N-Methyl-alpha-pyrrolidone	4.78	91	Bis(2-ethylhexyl) phthalate	19.41	83
	Pelargonic aldehyde	3.38	91	Bis(n-butyl) sebacate	7.87	91
	7H-Indolo[l, 2-b][2]benza-zepine, 8,9,10,12-tetrahydro-	3.19	64	Octadecenoic acid 2,3-dihydroxypropyl ester	6.64	58
	3-(p-Methylbenzoyl)-5,6-	3.09	64	alpha.-Gurjunene	3.33	81

	dihydro-4H -imidazo [1, 2-c] [1, 2 , 3] trizole					
	Di(n-butyl)sebacate	3.03	93	Butyl phthalate	2.88	72
	Azuleno [5,6-c]furan-1(3H)-one, 3a, 4,4a,5,6, 7,7a,8-octahydro-4-hydrox y-6,6,8-trimethyl-,	2.23	90	Azuleno[5,6-c]furan-1(3H)-one, 3a, 4, 4a,5,6, 7,7a,8-octahydro-4-hydrox y-6,6,8-trimethyl-	3.40	96
	Chrysorrheal	2.10	83	Oleic acid methyl ester	3.35	68
	Iso-velleral	1.65	51	Lactaroryfin A	1.52	47
	Velleral	1.59	44			
	Leaf aldehyde	1.41	53			
	Critonilide	4.94	83			
	Other 36 substances	46.05		*Other 12 substances*	16,24	
1.5	Stahlianthusone	7.08	93	2,4,6-Trimethyl-2-(4-methyl-pent-3 -enyl)-2H-pyran	7.33	38
	2-(4-Methoxyphenyl)-N,N,2-trimethyl-1-pyrrolamine	5.59	90	(1^1RS,2'RS,3^1SR)-3-(2^1,3'-epoxy-2' ,6',6'-trimethylcyclohexyl)-1-methyl-l-cyclobutene	26.61	90
	l-Methyl-2-pyrrolidinone	4.60	91	l-Methyl-2-pyrrolidinone	19.01	91
	Critonilide	4.94	83	2H-l-Benzopyran-5-carboxaldehyde	6.29	43
	Velleral	2.13	81	Humulen-(vl)	4.60	43
	Bis(2-ethylhexyl) phthalate	21.07	83	Bis(2-ethylhexyl) phthalate	10.20	80
	Dihydro-(-)-Neoclovene-(I)	26.11	70	Bis(n-butyl) sebacate	4.58	72
	6,7-Dimethyl-l,2,3,5,8,8a-hexahydronaphthalene	1.25	83	Azuleno[5,6-c]furan-1(3H)-one, 3^a, $4,4^a$,5,6,7, 7^a,8-octahydro-4-hydrox y-6,6,8-trimethyl-	5.54	86
	3-Amino-2´-nitrodiphenyl ether	1.36	91	1,4:5,8-Diethenobenzo [3,4]cyclobuta[1,2-a]cyclopropa[c]cycloheptene	10.87	83
	Other 12 substances	25.85		*Other 1 substances*	4.97	
1.6	Bis(2-ethylhexyl) phthalate	12,74	83	9-Octadecenamide	84.42	72
	N-Methyl-2-pyrrolidin	10,92	91	3-Nitrophthalic acid	4.24	59
	9-Octadecenamide	9.93	57	Bis(2-ethylhexyl) phthalate	2.28	83
	Isolantolactonoid butenolide A	5.95	66	Diisooctyl phthalate	3.36	72
	1-(1,1-dimethylethyl)-Naphthalene	4.21	89			
	4-methoxy-1,2,6,8-tetramethyl-Naphthalene	4.00	83			
	Velleral	3.40	55			
	Other 22 substances	48.85		*Other 9 substances*	5.7	

Note: Table does not include substances whose content in fractions is below 1%, and the degree of trustworthy is relatively low.

4. Conclusions.

It was found that the methylene chloride extracts obtained from fresh and dried fruit bodies of L. pergamenus mushroom differ significantly in both qualitative and quantitative composition. This difference was detected by TLC, GC-MS and the DPPH assay, and it is mainly caused by substances which are readily oxidized on the open air. The results of our study have demonstrated that the antioxidant activity of the methylene chloride extracts of fruit bodies of L. pergamenus mushrooms is provided by the velleral dialdehyde, azulene sesquiterpenes, leaf and pelargonic aldehydes, and amide of the oleic acid.

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REFERENCES

[1]. Gussem K.D., Verbeken A., Vandenabeele P., Gelder J.De, Moens L. 2006. Raman spectroscopic monitoring of Lactarius latex. Phytochemistry 67**,** 2580-2589. http://dx.doi.org/10.1016

[2]. Zaychenko O.I, Panchak L.V, Antonyuk V.O, Nemchenko O.O, Goschkina S.B, Tsivinska M.V, Stoika R.S, Kornijchuk O.P, Danylejchenko V.V 2010. *The ointment composition* for the external treatment of *the* human foot mycoses. Ukrainian patent № 54969. International Classes: A61K9/06, A61K35/84. Application Number: u 2010 08043. Filing Data: 20/07/2010. Publication Data: 25/11/2010 (in Ukrainian).

[3]. Panchak L.V, Klyuchivska O.Yu., Tsivinska M.V., Stoika R.S., Lesyk R.B., Antonyuk V.O 2011. Chemical composition of the frosen methanol extracts from true mushrooms fruit bodies. Biotechnology (Kyiv) 4**,** 90-96 (in Ukrainian). http://archive.nbuv.gov.ua/portal/chem_biol/Btl/2012_1/Panchak_1_2012.pdf

[4]. List PH und Hackenberg H 1969. Velleral und iso-Velleral, scharf schmeckende Stoffe aus Lactarius vellereus FRIES. Arch. Pharmaz. 302*:* 125-143*.* DOI: 10.1002/ardp.19693020208

[5]. Magnusson G and Thorén S 1973. Fungal Extractives. IV. Structure of a novel sesquiterpene dialdehyde from Lactarius by spectroscopic methods. Tetrahedron 29.1621 – 1624. http://dx.doi.org/10.1016

[6]. Daniewski W.M. and Vidari G, (1999) Constituents of Lactarius (Mushrooms).In Progress in the Chemistry of Organic Natural Products,Vol. 77*,* Part-III. Springer-Verlag; Wien-New York; 69-171. DOI 10.1007/978-3-7091-6366-5_3

[7]. Barros L, Baptista P, Ferreira IC. 2007. Effect of *Lactarius piperatus* fruiting body maturity stage on antioxidant activity measured by several biochemical assays**.** Food Chem. Toxicol*.* 45, 1731-1737. http://dx.doi.org/10.1016/j.fct.2007.03.006

[8]. Barros L, Baptista P, Estevinho IM, Ferreira IC 2007. Effect of fruiting body maturity stage on chemical composition and antimicrobial activity of *Lactarius* sp. mushrooms. J. Agric. Food Chem**.** 55, 8766-8771. DOI**:** 10.1021/jf071435+

[9]. Panchak LV and Antonyuk VO. (2011). Purification of a lectin from fruit bodies of *Lactarius pergamenus* (Fr.) Fr and studies its properties. Biochemistry (Moscow) 76. 438 – 449. DOI 10.1134/S0006297911040067

[10]. Antonyuk V.O, Lysyuk R.M, Antonyuk L.Ya. 2011. A practical course of pharmacognosy, Quart: Lviv. 499 P.

[11]. Kobulyanskuy E.V. 1996. A method of obtaining of a lubricant grease for polymeric materials. Russian patent № 2058301. International Classes: C07C233/09, C10M177/00, C10M105/68. Application Number: 5049085/04. Filing Data: 17/04/1992. Publication Data: 20/04/1996 (in Russian). http://ru-patent.info/20/55-59/2058301.html

[12]. Clericuzio M and Sterner O. 1997. Conversion of velutinal esters in the fruit bodies of Russula cuprea. Phytochemistry**,** 45. 1569-1572. http://dx.doi.org/10.1016/S0031-9422(97)00259-8

[13]. Rome D, Arzel E, Johansson M, Sterner O. 2008. Synthesis of biomimetic precursors of isovelleral analogues. Arkivog.*,* Part (vi), 91-99. https://www.arkat-usa.org/get-file/23444/

[14]. Touchstone JC. 1992. Practice of Thin Layer Chromatography, 3rd edition, Wiley-Interscience: New York.

[15]. Waksmundzka-Hajnos M, Sherma J, Kowalska T. 2008. Thin Layer Chromatography in Phytochemistry, Boca Raton - London – New York: CRC Press.

[16]. Asatiani MD, Elisashvili V, Wasser S.P, Reznick A.Z, Nevo E. 2007 Free-Radical Scavenging Activity of Submerged Mycelium Extracts from Higher Basidiomycetes Mushrooms. Biosci Biotechnol. Biochem 71. 3090 – 3092. http://dx.doi.org/10.1271/bbb.70280

[17]. Ferreira IC, Baptista P, Vilas-Boas M, Barros L. 2007. Free-radical scavenging capacity and reducung power of wild edible mushrooms from northeast Portugal: Individual cap and stipe activity. Food Chemistry 100**.** 1511 – 1516 http://dx.doi.org/10.1016

[18]. Keles A, Koca İ, Gençcelep H. 2011. Antioxidant Properties of Wild Edible Mushrooms. J Food Process Technol. 2. 2-6.doi:10.4172/2157-7110.1000130

[19]. Karunamoorthi K, Sabesan S*,* 2010. Laboratory evaluation of Dimethyl phthalate treated wristbands against three predominant mosquito (Diptera: Culicidae) vectors of disease Eur. Rev Med Pharmacol Sci. 14. 443-448