

BIOLOGICALLY ACTIVE SUBSTANCES OF METHANOL EXTRACTS OF DRIED *Lactarius quietus* AND *Lactarius volemus* BASIDIOMES MUSHROOMS: IDENTIFICATION AND POTENTIAL FUNCTIONS

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The aim of this investigation was to study chemical composition and biological effects of substances present in methanol extract of dried basidiocarps of *Lactarius quietus* and *L. volemus* and the action of substances of milky juice isolated from these fungi on the mammalian cells and invertebrate organisms. It was found that fresh juice of *Lactarius quietus* mushrooms reduced the viability of transformed mouse fibroblasts of L929 line and caused the death of the crustaceans (*Cyclops*) plankton. The extract from dried basidiocarps was fractionated by various organic solvents under the scheme described above for *Lactarius pergamenus* mushrooms. Obtained fractions were analyzed by gas chromatography combined with mass spectrometry. The largest range of compounds was present in the first (hexane) fraction containing substances whose analysis required additional separation on a silica gel column. The obtained fractions contained large amount of saturated and unsaturated higher fatty acids and their esters, phthalates and sesquiterpene compounds. Their composition and content in studied mushroom species differed significantly. The results of chemical analysis of mushroom basidiocarps allowed one to conclude that primary protective function in *L. quietus*, probably, was dependent on several sesquiterpene compounds of the azulene series — lactarorufin A and B. Since in *L. volemus* fungus the content of azulene and its derivatives is rather low, the biological activity of its milky juice towards the mammalian cells and small crustaceans is poorly studied.

Key words: *Lactarius pergamenus*, *L. quietus*, *L. volemus* mushrooms, methanol extraction, gas chromatography, mass spectrometry.

The basidiomycetes of the *Lactarius* genus differ from other fungi by presence of milky juice in the basidiocarps (fruiting bodies) which can play an important role in protecting them from damaging by insects, animals and destruction by the microorganisms. While young mushrooms contain a significant quantity of milky juice, in most cases, they are not wormy and slug, and forest rodents do not eat them. Such damaging was observed when fungi got senescent and the quantity of milky juice reduced. Thus, there are substances protecting those mushrooms present in the milky juice. These substances are not highly toxic to human and warm-blooded animals, and therefore, most fungi of the *Lactarius* genus are edible or conditionally edible. These

substances are of great interest because their study can help finding out how mushrooms protect themselves from damage. As shown in our previous studies [1, 2], these substances can be also used for medical purposes. It should be noted that substances of *Lactarius* milky juice are very unstable and significantly differ from substances present in dried mushrooms [3], however, they possess much higher biological activity and may participate in the defense of mushrooms against pathogens.

The aim of this study was to investigate and compare chemical composition of methanol extract of dried basidiocarps of *Lactarius pergamenus*, *L. quietus* and *L. volemus* and consider potential application of the individual compounds of such extract in biotechnology.

Materials and Methods

Fungi basidiocarps were harvested in July and August (2013–2014) during their mass appearance in the mixed forest of Skole district of Lviv region. Within 12 hours after collecting, mushrooms were brought to the laboratory and placed in the oven at 60 °C for 24–48 hours to complete drying.

Column chromatography on silica gel (Chemapol, Czech Republic) L40/160 mesh was used. Methanol, n-hexane, acetone, ethyl acetate, chloroform, isopropanol, n-butanol, diethyl ether were used as solvents and were qualified as «Pure for Analysis».

Fractionation based on using solvents and chromatography on silica gel

Dried basidiocarps of *Lactarius pergamenus*, *L. quetus* and *L. volemus* were crushed to powder ($d < 0.5$ mm), and then placed in the Soxhlet apparatus for 3–6 hours and extracted with methanol. Solvent was removed from the obtained extracts by distillation to small volume, and the remains of solvent was evaporated in the oven at 60 °C. The resulting dry residue was weighed and final extracts were used for studying their chemical composition.

Chemical composition of methanol fraction was investigated after separation into individual sub-fractions by a sequential extraction with organic solvents and water according to increasing polarity solvents (see previous work [2]). The solvent was distilled off and the obtained extract was dried at +56 °C. The residue was weighed and fractions were used for identification of their chemical composition.

It was shown that the fraction of methanol extract obtained from dried basidiocarps of *Lactarius pergamenus* possessed the most pronounced cytostatic effect towards murine leukemia L1210 cells comparing with fraction obtained by the extraction with hexane [2]. This fraction was composed of large quantity of substances whose identification required their prior separation. Taking into account that the same fractions of *L. quetus* and *L. volemus* might have similar structure, they were separated by column chromatography on silica gel and their chemical composition was examined by thin layer chromatography (TLC) and gas chromatography-mass spectrometry (GC-MS).

1.0 g of dark brown mass was obtained by the extraction with hexane of methanol and the residue was dissolved in 5.0 ml of n-hexane and applied on a column of silica gel L 40/160 (height — 40 cm and a diameter of 2.0 cm) which

was previously washed with pure n-hexane. The column was washed sequentially with the following solvent systems: n-hexane (20 mL), hexane-ethyl acetate 8: 1 (90 mL); hexane-ethyl acetate-methanol 4: 2: 1 (60 mL); hexane-ethyl acetate-methanol 2: 1: 2 (40 mL); methanol (60 mL). This made it possible to completely elute the pigment substances from the column. Fractions (1.5 ml) were collected in the pre-weighed tubes. The solvent was evaporated at 56 °C, fractions were weighed and a graph was built. According to the obtained results, 5 fractions were allocated from *L. quetus* and *L. volemus*. They were marked as fractions N 1.1 — N 1.5 and N 2.1 — N 2.5, accordingly. The analysis of these fractions was performed by GC-MS and some substances were identified by TLC on “Silufol” plate using appropriate solvent system and “witnesses” substances and substance-developers of the chromatograms. It was found that in most cases the obtained fractions consisted of different substances.

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

Chromatographic study of active substances by TLC on “Silufol” plates

The identification of certain substances was performed by thin layer chromatography on “Silufol” plates. The individual fractions obtained by such chromatography were applied onto the “Silufol” plate and chromatographed in the appropriate solvent system. After air drying, chromatograms were checked in ultraviolet light, after being sprayed with appropriate reagents used for identification of specific substances, and placed in a chamber with iodine crystals at the bottom.

To identify ergosterol, chromatography was performed in a system of hexane — ethyl acetate — methanol 40:6:1, by using as a “witness” the authentic sample of the ergosterol purified from baker’s yeast. Then the plate was sprayed with a saturated solution of tin chloride in the chloroform.

Higher fatty acids present in the fractions were discovered on the “Silufol” plates by chromatography of samples in the system hexane — ethyl acetate 8:1 or hexane — ethyl acetate-methanol 4:2:1. After chromatography, the plate was examined under UV light and processed by the iodine vapor, sprayed with a mixture of 0.1% methanol solution of the mixture of methyl red with blue brome thymol in order to identify free organic acids.

Study of the action of milky juice towards cultured cells

Milky juice was obtained from *L. pergamenus*, *L. quietus* and *L. volemus* basidiocarps of scarified selected fungi in the laboratory immediately prior to the experiment. Certain amount of the milky juice was weighed on the analytical balance and evaporated to determine the mass of solid residue. The transformed mouse fibroblasts of L929 line were grown in Dulbecco’s modified Eagle’s culture medium (DMEM, Sigma, USA), supplemented with 10% fetal bovine serum (Sangva, Ukraine) and 50 mg/ml of gentamycin (Sigma, USA). In order to determine the cytotoxicity, targeted cells were seeded in 24-well plates in DMEM supplemented with 10% of fetal bovine serum. After 24 hours of cell growing, 10 μ l of milky juice were added to 1 ml of culture medium. Cell number was counted at regular intervals in the hemocytometer chamber. Density of cell suspensions was calculated by the formula: $c = 12\,500\,n$, where c — number of cells in 1 ml of the suspension, n — average number of cells in 5 large squares of the chamber. The ratio of dead cells was determined after their staining 0.1% (W/V) solution trypan blue (Invitrogen, USA) and counting stained and unstained cells under light microscope Mik-Med 12 (LOMO, Russian Federation). After 2 days of cell culturing, changes in cell morphology were observed. Cells were stained with trypan blue and DNA-specific fluorescent dyes Hoechst 33342 (Merk) and the ethidium bromide (Sigma). Hoechst 33342 was added to cultured cells in final concentration of 1.0 μ g/ml, and cells were incubated for 20–30 min. The ethidium bromide was brought to a final concentration of ~0.5 μ g/ml immediately prior to cell observation under the fluorescent microscope (Mik-Med 12). During the microscopic study

of cell viability, the magnification was ~400–500 times at the relevant wavelength: Hoechst 33342 — excitation — 340 nm and emission — 450 nm; ethidium bromide — excitation — 510 nm and emission — 595 nm.

Study of the action of milky juice towards the planktonic crustaceans

The planktonic crustaceans — *Cyclops* sp. were collected in a marshy pond near Lviv city (Ukraine). The milky juice was obtained from *L. pergamenus*, *L. quietus* and *L. volemus* mushrooms. In control, the juice obtained from the basidiocarps of edible mushroom *Leccinum aurantiacum* (*Boletus aurantiacus* Bull. ex Fr.) on the *Cyclops* sp. was applied. 15%, 30% and 50% in water dilution of the milky juice were used. Five individuals of *Cyclops* sp. were placed in the diluted juice, and changes in the locomotor activity of this organism during 1, 5, 10, 15 and 20 min were monitored. After these intervals of time, the individual copepods were washed from the milky juice, and their return to normal motor activity was studied.

Statistical Analysis

All experiments were repeated at least three times and with minimum three parallels. Standard deviation was calculated and statistical significance of difference was evaluated by using Student’s t-test ($P < 0.05$).

Results and Discussion

In previous study [2], methanol extraction of the residue of *L. pergamenus* by the organic solvents was carried out in an ascending order of their polarity and 8 fractions were obtained. Using similar extraction procedure for *L. quietus* and *L. volemus*, one fraction less was obtained. The results of quantitative and qualitative analysis of the obtained fractions are presented in Table. 1.

The first (hexane) fraction isolated from *L. pergamenus* mushrooms contained the bulk of biologically active substances [2]. Probably, other fungi of the *Lactarius* genus in the analogical fractions contain a bigger panel of such active substances. Indeed, the quantity of this substances in hexane fractions was the highest in *L. volemus* (93%) and *L. quietus* (85.7%). The analysis of substances present in the hexane fractions by using GC-MS without further separation was not successful. That is why, a separation of hexane fractions of *L. volemus* and *L. quietus* was conducted by means of chromatography on silica gel (Fig. 1 and 2). The obtained chromatographic fractions were analysed by the GC-MS, and the results are presented in Table 2.

Table 1. Chemical composition of the methanol extract of basidiocarps *L. quetus* and *L. volemus* (based on the results of gas chromatography — mass-spectroscopy)

Frac-tion number	Extracting solvent	<i>L. quetus</i>		<i>L. volemus</i>	
		Amount	The main identified substances (% content in the fraction)	Amount	The main identified substances (% content in the fraction)
1	Hexane	857.0 mg	A complex mixture of substances (Table. 2)	930.0 mg	A complex mixture of substances (Table. 2)
2	Chloroform	16.0 mg	26 substances, the most important of which are: Lactarorufin B (34.06) Lactarorufin A (20.86) Stearic acid (14.17) Linolenic acid (10.50) Palmitinic acid (4.08) Erucylamide (2.01) Nicotinic acid (1.59) Trans valerenol (1.34) Gibberellin A3(0.66)	27.0 mg	13 substances, the most important of which are: Ergosta-7,22-dien-3-ol (63.70) Ergosta-5,7,22-trien-3-ol (23.85) Oleic acid (5.23) 10-octadecenoic acid methyl ester (1.42) (22E)-Ergosta -5,7,9 β (11) 22-tetraen-3-ol (1.70) Ergost-7-en-3-ol (1.70)
3	Diethyl ether	3.0 mg	Mixture of unanalysed substances	4.0 mg	4 substances, according GC-MS: Erucylamide (9.07) Ergosta-5,7,22-trien-3-ol (58.63) N-methyl adamantaneacetamide (29.25)
4	Ethyl acetate	21.0 mg	17 substances, the most important of which are: Lactarorufin B (49.99), Lactarorufin A (11.25) Glycerol (7.17) Stearic acid (3.69) Oleic acid (2.1) Nicotinic acid (0.91) Palmitinic acid (1.66)	10.0 mg	2 substances: Bis(2-ethylhexyl) phthalate (52.62) Bis(2-ethylhexyl) sebacate (47.38)
5	Butanol	42.0 mg	15 substances, the most important of which are: Lactarorufin B (49.95), Lactarorufin A (10.50) Glycerol triacetate (13.81) Glycerol (8.16) Glycerol monoacetate (1.52) Nicotinic acid (0.58)	1.0 mg	2 substances: Mannitol (50.11) Propantriol diacetate (40.89)
6	Isopropanol	27.0 mg	5 substances, the most important of which are: N-methyl-2- formylpyrrole (70.97) Lactarorufin A (11.67) Glycerol (10.78)	7.0 mg	Sorbitol (100.0)
7	Methanol	3.0 mg	3 substances: Mannitol (86.21) Oleic acid (7.31) Silicic acid diethyl bis (tri-methyl silyl) ester (6.48)	12.0 mg	2-formyl-1-methylpyrrole (100.0)

Note: *Italic font* was used in the Table to denote substances with less than 50 % reliability of GC-MS results. In brackets after the name of substances its amount (in %) in fraction is noted.

Table 2. Chemical composition of the hexane fraction basidiocarps *L. quetus* and *L. volemus*
(based on the results of gas chromatography-mass spectroscopy)

Fraction number	<i>L. quetus</i>		<i>L. volemus</i>	
	The main identified substances (% content in the fraction)	Degree of precision	The main identified substances (% content in the fraction)	Degree of precision
1	Total found 7 compounds, the main of which are: Ethyloleate (21.97) Linolenic acid (17.23) Isopropyl stearate (15.79) 9-octadecanoic acid (15.05) 3-Nitrophthalic acid (11.29)	99 93 90 68 83	Total found 11 compounds, the main of which are: Ethyloleate (37.93) Elaidic acid isopropyl ester (20.45) azulene (12.45) Linoleic acid ethyl ester (8.02) Isopropyl linoleate (5.76)	99 90 95 99 98
2	Total found 10 compounds, the main of which are: Ethyl linoleate (20.28) Ethyl oleate (19.63) Isopropyl linoleate (15.83) Isopropyl stearate (10.88) Oleic acid (10.59) Bis(2-ethylhexyl) phthalate (9.09) Isopropyl palmitate (5.06) Stearic acid (3.38) Ethyl palmitate (2.77) Dibutylphthalate (2.49)	83 99 99 91 93 87 91 99 98 83	Total found 22 compounds, the main of which are: Oleic acid (90.54) n-Hexadecanoic acid (4.38)	95 98
3	Total found 14 compounds, the main of which are: Linolenic acid (43.73) Stearic acid (36.02) Palmitic acid (17.79) Pentadecanoic acid (1.17)	99 99 99 99	Total found 12 compounds, the main of which are: Oleic acid (45.35) 9,12-Octadecadienic acid (43.6) 2-Methylphenyl hydrazine (2.36) 3,3-Dimethylheptanoic acid (2.02) n-Hexadecanoic acid (1.52) 9-Octadecanoic acid 2-hydroxy (1-hydroxy) methyl ester (1.14)	94 94 45 86 99 55
4	Total found 10 compounds, the main of which are: Oleic acid (30.24) Stearic acid (20.27) Ergosterole (18.76) Linolenic acid (10.35) Palmitic acid (5.31) 1-Bromo-4,8 dimethyl-3(E) 7-nonadiene (3.91) 9-octadecenamide (3.23) Bis(2-ethylhexyl) phthalate (3.20)	96 99 89 99 99 35 58 83	Total found 6 compounds, the main of which are: Ergosterole (57.06) 5,6-Dihydroergosterole (27.98) 2-hydroxycyclopentadecanone (5.56) 7-chloroquinoline 2,4-dicarboxylic acid (4.02)	93 97 64 47
5	Total found 52 compounds, the main of which are: Lactarorufin A (17.85) Glycerol-1-monooleate (14.05) Oleic acid (5.38) Glycerol (4.29) 1,3,4,4a- β , 5,6,8,9-octahydro-4a-hydroxy-6,6,8 β -trimethylazuleno(5,6-c)-furan-2-one (3.89) 9-Phenanthrenol (3.42) 7-Pentadecyne (2.83) Linolenic acid (2.32) Linolic acid (1.96)	93 94 99 83 87 90 93 97 99	Total found 41 compounds, the main of which are: 9-octadecenal (17.25) Oleic acid (14.45) Phtalic acid (5.23) Phenylacetic acid (4.64) 9,12-octadienic acid (3.89) Glycerol (3.25) Octadecenic acid (3.13) Iridomyrmecin (3.03)	95 93 97 87 93 78 97 45

Notes. The table does not include the compounds whose content in fractions is less than 1% and the degree of precision of identification is lower than 50%. In the parentheses after the name of the name compounds their content in % in the fractions is noted.

* The degree of precision in the identification of substances defined as a percentage of coincidence mass spectra of the reference material in a computer database and substances in the analyzed fractions. The calculations were done using computer software mass-spectrometer.

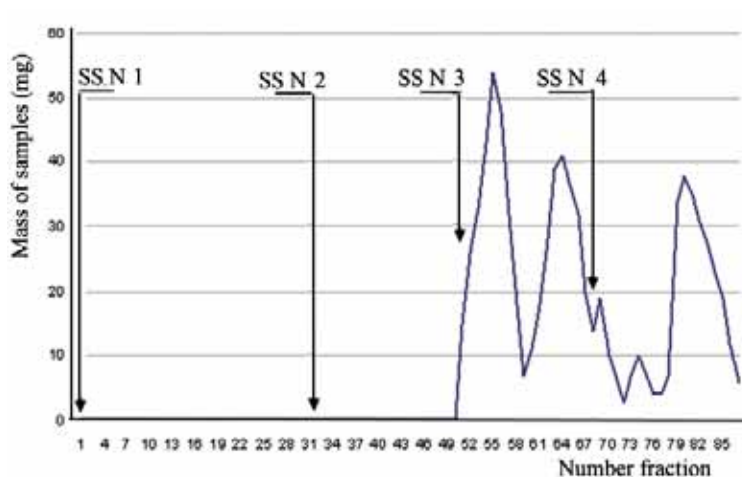


Fig. 1. Chromatogram of separation of hexane fraction of the methanol extract of *L. volemus* on a column of the silica gel ($h = 40$ cm, $d = 2.0$ cm):

SS (solvent system) N 1 — hexane-ethyl acetate 8: 1;

SS N 2 — hexane-ethyl acetate-methanol 4: 2: 1;

SS N 3 — hexane-ethyl acetate-methanol 2: 1: 2;

SS N 4 — methanol.

Fractions N 1.1–1.5 were formed using combined eluate in test tubes: N 1.1 — 51–59; N 1.2 — 60–68; N 1.3. — 69–72; N 1.4. — 73–77; N 1.5. — 78–87

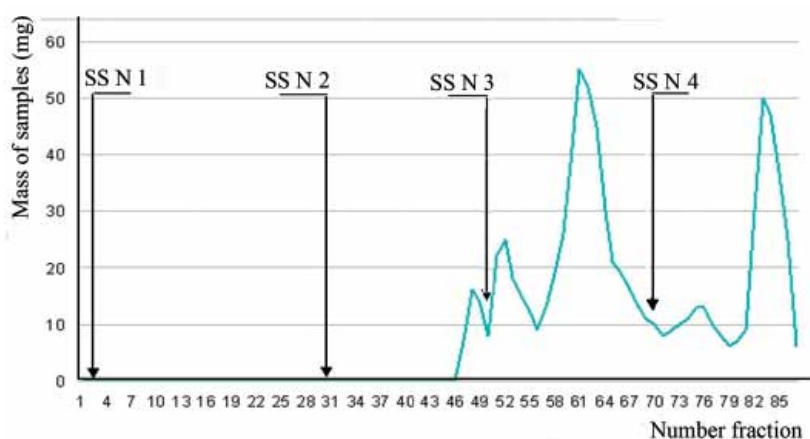


Fig. 2. Chromatogram of separation of hexane fraction of the methanol extract of *L. quietus* on a column of the silica gel ($h = 40$ cm, $d = 2.0$ cm):

Solvent system (SS) — the same as in Fig. 1. Fractions N 2.1–2.5 were formed using combined eluate in test tubes: N 2.1 — 47–50; N 2.2 — 51–56; N 2.3 — 57–73; N 2.4 — 74–80; N 2.5 — 81–87

Comparison of chemical composition of similar fractions of the *L. pergamenus* fungus [2] and *L. quietus* and *L. volemus* fungi (this study) is presented in Table 1 and 2 and demonstrates a significant difference between them. It was found that, namely in *L. pergamenus* most of sesquiterpene compounds are concentrated in hexane fraction of the methanol extract, while the chloroform fraction contains much less of these compounds. The chloroform fraction of the methanol extract of *L. quietus* contained $\approx 55\%$ of lactarorufin A and B sesquiterpenes,

while the analogical fraction of *L. volemus* contain 90% of ergosterol. The lactarorufin A and B were first found in the morphologically similar *Lactarius rufus* fungus [4], in which they accounted for more than 50% in the ethyl acetate and butanol fractions of the methanol extract of *L. quietus*. These substances were detected in *L. pergamenus* and *L. volemus*. Butanol and isopropanol fractions of *L. volemus* mainly contain mannitol and sorbitol, whereas similar fractions of *L. pergamenus* and *L. quietus* contain a large amount of glycerol and its esters. In *L. pergamenus* and *L. quietus* fungi,

mannitol is present in methanol (*L. quetus*) and water (*L. pergamenus*) fractions. While in *L. quetus* and *L. volemus*, final extraction with water did not extract any substances, but in *L. pergamenus*, water fraction contains 48.3% of substances of the methanol extract.

Separation of hexane fraction of the methanol extract of *L. quetus* and *L. volemus* on a column of silica gel using the same sequence of solvents gave different results. Applying hexane-ethyl acetate 8: 1 system towards *L. pergamenus* permitted elution of three fractions of substances [2], while similar treatment of *L. quetus* and *L. volemus* on a column of silica gel did not elute them. In these fungi, separation into sub-fractions occurred only after addition methanol to solvent system.

A question appears — why some substances are located in several fractions at using different solvents and various chromatographic separations. Higher fatty acids and their esters belong to these substances. The reason for that could be mutual dissolubility of substances of the milky juice of mushrooms, as well as formation of ternary systems that are difficult to separate. Thus, at analyzing hexane fraction of the methanol extract of *L. pergamenus*, fractions 1.4 can be successfully analyzed only by using GC-MS and further chromatographic separation on a column of silica gel L 40/160 in the elution system of chloroform — dioxane — methanol with changing the ratio of these solvents. In this case, applying 1,4-dioxane which mixes well with polar and non-polar solvents (hexane, chloroform, methanol and water) plays a key role [5].

The heterogeneity of substances in the corresponding extracts of fungi was also confirmed by the results of chromatography on the “Silufol” plates. For example, fraction 1.4 of hexane fraction of the methanol extract of *L. volemus* contains 85% mixture of ergosterol and 5.6-dihydroergosterol (Table 2). Chromatography of this fraction in comparison with the sample baker’s yeast ergosterol, followed by detection of chromatograms with tin chloride in chloroform after 30 min, revealed dark purple spots with the same value R_f , as in the control (ergosterol) sample (Fig. 3).

High content of the organic acids (Table 2) in fractions of 1.2–1.3 of the hexane extract of *L. volemus* and in a similar fraction 2.3. of *L. quetus* was confirmed by the TLC followed by using a mixture of 0.1% methanol solution of the methyl red and brome thymol blue. Chromatographic processing with iodine vapor permitted detecting a variety of organic

substances. Comparison of the results obtained with the GC-MS (Table. 2) of five fractions of the hexane extract of *L. quetus* (Fig. 4), permitted to conclude that the iodine reacts with unsaturated organic acids (eg, oleic acid, linoleic and), as well as with some substances that are easily oxidized. It is considered that chromatography in a thin layer sorbent is less precise than the GC-MS identification of the organic compounds and determination of their quantitative content. Thus, TLC can play only a supporting role, particularly, in doubtful cases which require identification of certain substances.

Action of milky juice of mushrooms towards cultured murine fibroblast cells of L929 line

Milky juice of all three fungal species contained $\approx 10\%$ of dry matter. Its concentration in the medium during cultivation of transformed murine fibroblasts of L929 line was 1 mg/ml. The most remarkable effect towards these cells was observed under the action of substances present in *L. quietus* milky juice (Fig. 4), while the substances present in the milky juice of other fungus — *L. volemus* belonging to the same genus did not significantly influence the cells (data not shown). A big part of cells were affected by substances of the milky juice of *L. quietus*, and cells with altered morphology and giant size were observed. When a combined staining of cells by Hoechst 33342 (blue) and ethidium bromide (red) was used, nucleus of a pink color and a fraction of cells with red nucleus characteristic for the necrotic cells were also observed. Thus, substances present in the milky juice of *L. quietus* increased the permeability of the plasma membrane of target cells (Fig. 4). However, the relative number of cells stained by the trypan blue was low, which suggests that a majority of the treated cells retained their viability.

Action of milky juice of studied mushrooms towards the crustacean (Cyclops) Plankton

Five minute action of 15% solution of milky juice of all *Lactarius* species and of juice orange-cap boletus mushroom (*Leccinum aurantiacum* (Bull. Ex Fr.) SF Gray) did not cause a decrease in motor activity of the *Cyclops*. 50% extract of *Leccinum aurantiacum* also did not change the locomotor activity of *Cyclops*, while the *Lactarius* milky juice in such concentration caused a significant reduction in the motor activity of *Cyclops* that resulted in weak movements of their limbs and antennae. The biggest impact was observed in

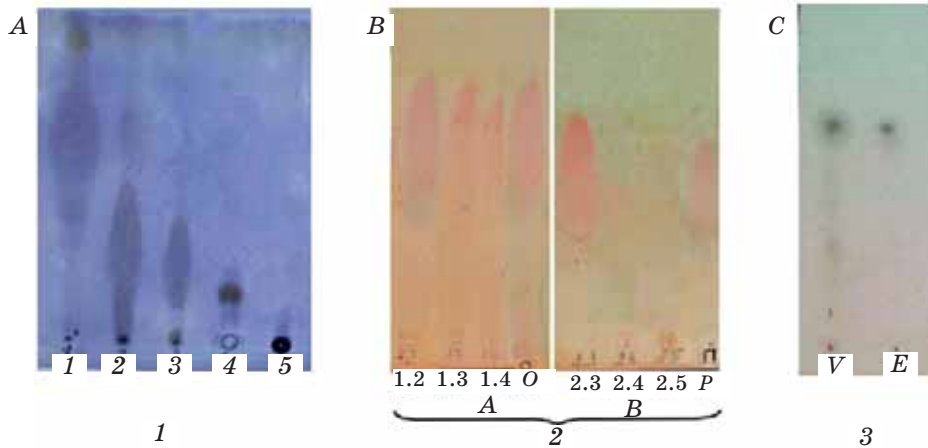


Fig. 3. Thin layer chromatogram of the lipophilic substances of extracts of *L. quietus* and *L. volemus* (“Silufol” plates) in solvent system:

- 1 — treatment with iodine vapor of five fractions of the hexane extract of *L. quietus*;
- 2 — treatment with a mixture of 0.1% methanol solution of the methyl red + brome thymol blue (identification organic acids). A — hexane extract fraction 1.3–1.5 *L. volemus*; B — hexane extract fraction 2.3–2.5 *L. quietus*, O — oleic acid, P — palmitic acid;
- 3 — chromatography fractions 1.4 hexane extract of *L. volemus* (V) and ergosterol baker’s yeast (E). Development of chromatograms was achieved by saturated solution of tin chloride in the chloroform

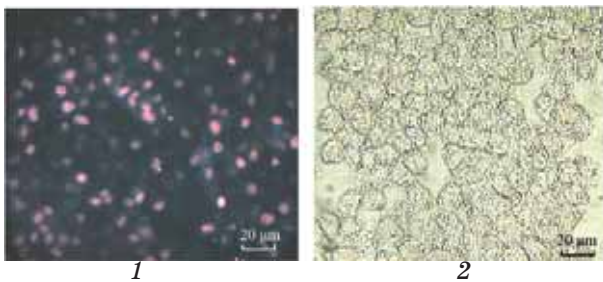


Fig. 4. Ethyidium bromide and Hoechst 33342 dye staining of transformed murine fibroblasts of L929 line after their exposure to substances of the milky juice of *L. quietus* mushroom:

- 1 — fluorescence microscopy;
- 2 — light microscopy

the case of using *L. quietus* juice which caused death of all individuals of *Cyclops* as soon as in 5 min of treatment. In 50% juice of *L. pergamenus*, death of the copepods was observed in 10 min, while in case of applying *L. volemus* juice — in 15 min. 100% juice of *Leccinum aurantiacum* did not cause death of the *Cyclops* even at 20 min treatment, while the *Cyclops* survived for only 5 min in juice of *L. volemus*. The *L. pergamenus* juice killed the *Cyclops* as soon as in 1 min, and the juice of *L. quietus* did that immediately.

Taking in account these results, we suggested that the milky juice isolated from *L. pergamenus*, *L. quietus* and *L. volemus* basidiocarps contains specific substances affecting the locomotor activity of the aquatic crustaceans, and the action of those substances

depended on its duration. The highest toxicity was observed in case of using the milky juice of *L. quietus* mushroom. Washing with water of *Cyclops* after a short exposure (1 min) eliminated substances present in the milky juice and normalized motor activity of the copepods.

It was established that drying of mushrooms led to a significant change in their chemical composition, apparently, as a result of action of high temperature and enzymatic reactions that can occur during the drying process. In dried mushrooms, labile substances disappear and stable compounds appear. We have demonstrated that drying practically eliminated the burning taste of the *L. pergamenus* mushrooms caused by substances of aldehyde nature preventing the mushrooms against eating by the shellfish and small rodents. The milky juice of *L. quietus* and *L. volemus* does not possess a burning taste, however in young age, these fungi are very rarely damaged by the mollusks and rodents. This suggests that mentioned mushrooms also contain certain substances playing a protective role. Drying of mushrooms destroys a significant amount of these substances, thus, only stable substances (regarding the biological effects) are of practical interest. Previously, dried basidiocarps of *L. pergamenus* containing 3,14,15-trimethylfuranolactaran-8-ol were proposed for treatment of foot mycoses in humans [1]. Thus, not only fresh mushrooms,

but also dried ones can be of practical significance.

Obviously, the main biological significance of the milky juice of *Lactarius* basidiocarps is based on protecting the fungus from a variety of pathogens and from eating by animals. The results of our studies demonstrated that juice of three fungi significantly differ in its chemical composition and organoleptic properties. The milky juice of *L. pergamenus* is very caustic and burning, while the juice of *L. quetus* and *L. volemus* does not taste like that. These mushrooms differ in quantity of milky juice and content of sesquiterpenes, however, they are all well protected against their pests.

The milky juice of studied mushrooms contains at least two groups of substances: 1) substances curing stability of juice emulsion; 2) substances possessing a protective effect. The stability of these emulsions is caused by the higher fatty acids and their esters which were found in large quantities in the milky juice of all three fungi species. In the milky juice of *L. pergamenus*, high content of the stearic acid was detected. Most likely, this is due to formation of the stearates of the sesquiterpene compounds. It was shown that the milky juice of *L. pergamenus* contains sesquiterpenes of the marasmane group, namely the velutinal stearate which along with 6-ketostearyl velutinal, was also found in *L. vellereus* and *L. necator* [6, 7]. Perhaps, other higher fatty acids form complexes with sesquiterpenes present in juice of these fungi, or are available there in a free state serving as the emulsifiers. It should be noted that high content of fatty acids was also found in some other fungi species of the *Lactarius* genus, particularly in *L. deliciosus* [8].

In *L. quetus*, basic protective function is likely performed by sesquiterpene compounds of azulene series — lactarorufin A and B that are found in the lipophilic extracts of dried basidiocarps. Thus, sesquiterpenes of different chemical structure are major protective compounds in many species of fungi of the *Lactarius* genus. They express antimicrobial, antifungal and insecticidal activity [9, 10].

In *L. volemus*, azulene is present in the hexane fraction, although its quantity there is relatively small. A protective role against pathogens might be played by 7-chlorohinolin-2.4-dicarboxylic acid, phthalic and phenylacetic acid that are present in the juice of this fungus and possess the antibiotic

activity [11, 12]. High ergosterol content was detected in the milky juice of *L. volemus*. In general, the biological activity of the milky juice of *L. volemus* was lower than that in *L. quetus*. Perhaps, a protective effect of the milky juice of *L. volemus* can explain its high content in young mushrooms. At damaging freshly basidiocarps of *L. volemus* one can observe an intense leakage of juice of this fungus which has also a Ukrainian name of “pidmolochnyk”, while the injury of *L. quetus* leads to a weak production of the latex. A question appears about practical significance of the labile substances of fungi of *Lactarius* genus. It is known that young fungi of this genus are filled up with latex and, thus, they are practically not damaged by worms, microorganisms or eaten by animals. There is a number of works of different authors showing high antimicrobial activity of substances present in the milky juice of mushrooms [13–15]. These authors also noted instability of such substances and the dependence of the antimicrobial activity on the stage of fungi development [14]. Such substances are difficult to obtain by conventional chemical methods. Thus, biotechnological methods using immobilized fungi cells or their callus culture can be used for synthesis of these specific substances of the milky juice.

Here we studied chemical composition of dried basidiocarps of three species of fungi of the *Lactarius* genus — *L. pergamenus*, *L. quetus* and *L. volemus*. It was shown that their chemical composition differs significantly. However, they are similar regarding presence of the milky juice in fresh mushroom basidiocarps. To secure stability, special substances acting as juice emulsifiers are necessary and this function can be performed by higher fatty acids and their esters. For protection against pathogens and eating by animals, different organic compounds are present in fungi juice. They are insoluble in water and, therefore, present in the emulsified state. These compounds belong to azulene derivatives of sesquiterpenes (*L. quetus* and *L. volemus*) or marasmane derivatives (*L. pergamenus*) [3, 5]. In addition, these fungi contain substances of other chemical nature that may protect from the action of other groups of pathogens. These are phthalates, quinoline derivatives and some other substances.

In previous works, we have studied chemical composition of dried [2, 5] and fresh [3] basidiocarps of *L. pergamenus*. It was shown that their chemical compo-

sition differed significantly. Many labile substances of the milky juice become more stable but less biologically active after drying of the basidiocarps. It is also accompanied by a reduction of their antioxidant properties [3] or and their antifungal action [1]. These labile compounds are of big interest for medicine, biotechnology and agriculture. For example, protection of crops from eating by insects and their larvae are important for increasing crops productivity. These compounds can be

also used as biological and biotechnological tools [16]. Labile substances of the milk juice of fungi of *Lactarius* genus are of particular interest. Antifeedants formed in case of damaging basidiocarps of these mushrooms, can scare various rodents by burning or unpleasant taste. However, these compounds are rapidly converted to inactive and harmless for human state.

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**БІОЛОГІЧНО АКТИВНІ РЕЧОВИНИ
МЕТАНОЛЬНОГО ЕКСТРАКТУ
ВИСУШЕНИХ БАЗИДИОМ ГРИБІВ
Lactarius quietus I *Lactarius volemus***

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Метою роботи було дослідити хімічний склад і біологічну дію метанольного екстракту висушених базидіом *Lactarius quietus*, *L. volemus* і молочного соку цих грибів на клітини ссавців та організм безхребетних. Встановлено, що свіжий сік грибів *Lactarius quietus* знижує життєздатність трансформованих мишачих фібробластів лінії L929 і спричинює загибель ракоподібних (циклопи) планктону. Екстракт висушених базидіом фракціонували різними органічними розчинниками за схемою, описаною нами раніше для *Lactarius pergamenus*. Фракції аналізували газовою хроматографією — мас-спектрометриєю. Найбільшим асортиментом сполук характеризується перша (гексанова) фракція, що містить речовини, для аналізу яких необхідним було додаткове розділення на колонці силікагелю. Отримані фракції містили велику кількість насичених і ненасичених вищих жирних кислот і їх ефірів, сесквітерпенових сполук і фталатів. Їхній склад і вміст у грибах досліджуваних видів суттєво відрізняється. За результатами аналізу хімічного складу базидіом грибів зроблено висновок про те, що основну захисну функцію у *L. quietus*, наймовірніше, виконують сесквітерпенові сполуки азуленового ряду — лактарорурфин А і В. Оскільки у гриба *L. volemus* азулен та його похідні містяться у значно меншій кількості, біологічна активність його молочного соку щодо клітин ссавців і дрібних ракоподібних є менш вираженою.

Ключові слова: гриби *Lactarius pergamenus*, *L. quietus*, *L. volemus*, метанольна екстракція, газова хроматографія, мас-спектрометрія.

**БИОЛОГИЧЕСКИ АКТИВНЫЕ ВЕЩЕСТВА
МЕТАНОЛЬНОГО ЭКСТРАКТА
ВЫСУШЕННЫХ БАЗИДИОМ ГРИБОВ
Lactarius quietus II *Lactarius volemus***

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Целью работы было исследовать химический состав и биологическое действие метанольного экстракта высушенных базидиом *Lactarius quietus*, *L. volemus* и млечного сока этих грибов на клетки млекопитающих и организм беспозвоночных. Установлено, что свежий сок грибов *Lactarius quietus* снижает жизнеспособность трансформированных мышинных фибробластов линии L929 и вызывает гибель ракообразных (циклопы) планктона. Экстракт высушенных базидиом фракционировали различными органическими растворителями по схеме, описанной нами ранее для *Lactarius pergamenus*. Фракции анализировали газовой хроматографией-масс-спектрометрией. Наибольшим ассортиментом соединений характеризовалась первая (гексановая) фракция, содержащая вещества, для анализа которых было необходимо дополнительное разделение на колонке силикагеля. Полученные фракции содержали большое количество насыщенных и ненасыщенных высших жирных кислот и их эфиров, сесквитерпеновых соединений и фталатов. Их состав и содержание в грибах исследуемых видов существенно отличался. По результатам анализа химического состава базидиом грибов сделан вывод о том, что основную защитную функцию в *L. quietus*, вероятнее всего, выполняют сесквитерпеновые соединения азуленового ряда — лактарорурфин А и В. Поскольку у гриба *L. volemus* азулен и его производные содержатся в значительно меньшем количестве, биологическая активность его млечного сока относительно клеток млекопитающих и мелких ракообразных менее выражена.

Ключевые слова: грибы *Lactarius pergamenus*, *L. quietus*, *L. volemus*, метанольная экстракция, газовая хроматография, масс-спектрометрия.