Background. According to the World Health Organization antibiotic resistance is among the top ten threats to human health, food safety and development. Today antibiotic resistance has reached alarmingly high levels all over the world. Meanwhile, the increase in the synthetic drugs’ production has led to the pathogenic mycobacteria’s rapid adaptation to the created chemicals, which have a narrow focus of application. That is why in modern biotechnology and pharmacology much attention is paid to natural producers of biologically active compounds, in particular – to xylotrophic fungi. It has been experimentally proven that the xylotrophic macrocytome Fomitopsis officinalis or tinder fungus can be considered to be a promising producer of pharmacological substances with a broad spectrum of action. Studies of active metabolites, contained in the mycelial mass, culture fluid of the medicinal xylotrophic macrocytome F. officinalis, and determination of their biological action remain relevant.

Objective. The objective was to determine the antimicrobial activity of culture fluid and mycelial mass of F. officinalis different strains from the mushrooms collection (IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany, NAS of Ukraine) against gram-negative and gram-positive bacteria species.

Methods. An in vitro study of the antimicrobial activity of ethyl extract of extracts of culture fluid and aqueous-ethyl extracts of mycelial mass for F. officinalis strains IBK-5004, IBK-2497, IBK-2498 against gram-positive Staphylococcus aureus (B-918), Bacillus subtilis (B-901) and gram-negative Escherichia coli (B-906), Bacillus subtilis (B-900), Klebsiella pneumoniae (M-123) bacteria by disc-diffusion method was conducted.

Results. High antimicrobial activity of tinder fungus culture fluid and mycelial mass extracts against Staphylococcus aureus was established after the 21st day of cultivation, while on the 28th day the zone of growth retardation was maximal (15–25 mm). The highest indices were recorded in F. officinalis IBK-5004 (20–25 mm) and IBK-2498 (20–24 mm) strains. Antimicrobial activity against Klebsiella pneumoniae in culture fluid extracts was manifested on the 21st and 28th days of cultivation. The highest antimicrobial activity against Klebsiella pneumoniae was observed in the culture fluid of the strain F. officinalis IBK-5004, the diameter of the growth retardation zone was 18 mm on the 28th day of cultivation. Mycelial mass’s extracts showed moderate activity on the 14th day of cultivation (7-8 mm); maximal activity was recorded on the 28th day (12–22 mm). The most active strain was Fomitopsis officinalis IBK-2498. No antimicrobial activity against test organisms was detected in the following studied strains: Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis.

Conclusions. It has been established that the mycelial mass and culture fluid extracts of F. officinalis IBK-5004, IBK-2497, IBK-2498 strains have high antimicrobial activity against Staphylococcus aureus and moderate antimicrobial activity against Klebsiella pneumoniae on the 21st and 28th day of cultivation.

Keywords: antibiotic resistance; biologically active substances; mycelium; mycelial mass; culture fluid; gram-negative bacteria; gram-positive bacteria; disk diffusion method; anti-microbial activity.

Introduction

Research and development of new effective biological products for the treatment and prevention of acute and chronic diseases caused by various microorganisms is one of the priorities of modern pharmaceutical mycology. In recent decades, considerable attention has been attracted to antibiotic-producing fungi, which they synthesize in the process of secondary metabolism [1–6]. Synthesis of antibiotics is one of the forms of fungi antagonism against other organisms’ species. The antibiotics formation is evolutionary and adaptive in nature and is closely related to the general metabolic processes in fungal cells. In recent years, the increase in the production of synthetic drugs has led to the rapid adaptation of pathogenic mycobacteria to the developed chemicals, which have a narrow focus on application, and as a consequence, a significant number of adverse reactions for the human body. Prolonged and not always justified antibiotics application often accelerates the...
pathsogens evolution in the direction of consolidating their resistance to these drugs. Therefore, it is necessary to replace constantly some types of antibiotics with others. To do this, you need to find the most active organisms — antibiotics producers. That is why in modern medicine and pharmacology much attention is paid to natural producers of biologically active compounds, including xylotrophic macrofungi [1, 3, 7–10]. This is due to the fact that natural compounds from medicinal macrofungi, in contrast to synthetic, have a multifunctional and multifaceted effect on the human body, which significantly reduces the negative effects and addiction. The study of therapeutic activity of medicinal mushrooms different species has shown the feasibility of their application in modern clinical practice [3, 5, 11]. Increased attention to xylotrophic macrofungi is due to the fact that it has been experimentally confirmed that these organisms synthesize secondary metabolites of extremely diverse chemical structure, a significant proportion of which are inhibitors of various cellular processes. Such substances include antibiotics, fungicides, cytostatic compounds, modulators of the immune response, growth regulators. The synthesis of secondary metabolites with antimicrobial activity is more characteristic of wood-destroying basidiomycetes — wood brown rot putrefaction agents. One of such species is the valuable rare macrofungus *Fomitopsis officinalis* (Vill.) Bondartsev & Singer, known in medical practice as “tinder fungus” or “larch sponge”. The healing properties of larch sponge substances have been known for a long time and are widely used in traditional Chinese and Tibetan medicine [12–15]. Modern research has shown that larch sponge can be considered a promising producer of pharmacological substances with a broad spectrum of action. Unsaturated fatty acids (palmitic, oleic, linoleic, linolenic, arachidonic, etc.), heteropolysaccharides, glucosamines, agaric acid, phospholipids, carotenoids, sterols, vitamins of B group, vitamins E, A, essential oils, cytokinins, triterpenoids of lanostan type (eburic acid) were isolated from the culture fluid after growing the mycelium was separated from the biomass. Ethyl acetate (2:1 by volume) was added to the culture fluid to concentrate the antimicrobial substances, and the mixture was shaken vigorously for 10 min and left for 20 h at a temperature of 4 °C. Then the ethyl acetate layer was removed, evaporated on a rotary evaporator to dryness. The precipitate was dissolved in 70% ethanol. The solution of the obtained concentrate (10 μl) was applied to standard disks of Bio Merieux firm (6 mm in diameter), dried at 40 °C for 30 min, and placed on the surface of Mueller–Hinton agar seeded with test culture.

Taking into account the amount of concentrate applied to the disk in each sample was 0.10 mg of biologically active substances.

The mycelial mass was dried to constant weight at a temperature of 60 °C. Aqueous-alcoholic 70% extract was prepared at the rate of 20 mg of mycelial mass per 1 ml of solvent. The mycelial mass was crushed, the extraction was performed on an ultrasonic bath at a temperature of 40 °C for 30 min, left for a day in a refrigerator at a temperature of 4 °C, then filtered, centrifuged for 20 min at 13500 g.

**Materials and methods**

The objects of the study was pure cultures of *Fomitopsis officinalis*, which are stored in the IBK Mushroom Culture Collection of the M.G. Kholodny Institute of Botany of the National Academy of Science of Ukraine (Table 1).

**Table 1: The studied collections of Fomitopsis officinalis** [25]

<table>
<thead>
<tr>
<th>IBK number</th>
<th>Origin and date of culture isolation</th>
</tr>
</thead>
<tbody>
<tr>
<td>IBK-2497</td>
<td>Mycoforest Collection of typical cultures (MFTCC), Slovakia, 2016</td>
</tr>
<tr>
<td>IBK-2498</td>
<td>Mycoforest Collection of typical cultures (MFTCC), Slovakia, 2016</td>
</tr>
<tr>
<td>IBK-5004</td>
<td>V.L. Komarov Botanical Institute, Russian Academy of Science (BIN), St.-Petersburg, Russia, 1981</td>
</tr>
</tbody>
</table>

**Preparation of mushroom extract.** *F. officinalis* cultures were grown superficially on a complex nutrient medium GPE, of the following composition, g/l: glucose — 25.0; peptone — 3.0; yeast extract — 2.0; KH₂PO₄ — 1.0; K₂HPO₄ — 1.0; MgSO₄.7H₂O — 0.25. The acidity of the medium was 5.5. Cultivation was performed at a temperature of 26 ± 1 °C for 7, 14, 21, and 28 days. The culture fluid after growing the mycelium was separated from the biomass. Ethyl acetate (2:1 by volume) was added to the culture fluid to concentrate the antimicrobial substances, and the mixture was shaken vigorously for 10 min and left for 20 h at a temperature of 4 °C. The ethyl acetate layer was removed, evaporated on a rotary evaporator to dryness. The precipitate was dissolved in 70% ethanol. The solution of the obtained concentrate (10 μl) was applied to standard disks of Bio Merieux firm (6 mm in diameter), dried at 40 °C for 30 min, and placed on the surface of Mueller–Hinton agar seeded with test culture.

The mycelial mass was dried to constant weight at a temperature of 60 °C. Aqueous-alcoholic 70% extract was prepared at the rate of 20 mg of mycelial mass per 1 ml of solvent. The mycelial mass was crushed, the extraction was performed on an ultrasonic bath at a temperature of 40 °C for 30 min, left for a day in a refrigerator at a temperature of 4 °C, then filtered, centrifuged for 20 min at 13500 g.
**Bacterial test organisms.** Daily bacterial cultures from the Collection of the Department of Biotechnology and Microbiology of the National University of Food Technologies (Kyiv, Ukraine) were used as test cultures: *Staphylococcus aureus* (B-918), *Pseudomonas aeruginosa* (B-900), *Escherichia coli* (B-906), *Bacillus subtilis* (B-901), *Klebsiella pneumonia* (M-123), which were pre-grown in tubes on Mueller–Hinton slope agar medium (Oxoid). Several same-type clearly isolated colonies of bacteria were selected for inoculum preparation. A small amount of material from the tops of the colonies was transferred by microbiological loop into a test tube with sterile physiological saline, shaken to obtain a homogeneous suspension, bringing the inoculum density to exactly 0.5 according to the McFarland standard (5·10^6 cells/ml) Mc Farland (No: 0.5) standard. Use the inoculum within 15 min after preparation. A suspension of bacteria in an amount of 0.2 ml was evenly applied to the surface of the Mueller–Hinton agar medium (Oxoid).

**Performing of antibacterial screening test.** In the study of mycelial mass and culture fluid antibiotic activity, disk diffusion method (DDM) was used [11]. Standard sterile disks were impregnated with extract samples, placed on the surface of Mueller–Hinton agar seeded with test culture. The cultures were incubated at 37°C for 24 h. Next, the zone of microorganisms' growth inhibition was determined. The results were evaluated by the diameter of the growth retardation zones around the disk: no growth retardation zone – the test culture is not sensitive to this specimen concentration; the diameter of the growth retardation zone is less than 10 mm – moderate sensitivity of culture to the given specimen concentration; the diameter of the growth retardation zone is more than 10 mm – high sensitivity of the test culture to this specimen concentration.

Gentamycin sulphate (40 mg/ml), Ukraine, was used as a positive control. Gentamycin sulphate is a broad-spectrum aminoglycoside antibiotic. It has a bactericidal effect. Actively penetrating the cell membrane of bacteria, it irreversibly binds to the 30S subunit of bacterial ribosomes and, thus, inhibits the synthesis of the pathogen protein. In vitro tests confirmed its high activity against aerobic gram-negative bacteria: *Escherichia coli*, *Klebsiella spp.*, *Pseudomonas aeruginosa*, *Shigella spp.*, *Salmonella spp.*, *Enterobacter spp.*, *Serratia spp.*, *Proteus spp.*, *Acinetobacter spp.*. It is also active against aerobic gram-positive cocci: *Staphylococcus* spp. (including resistant to penicillins and other antibiotics), some strains of *Streptococcus* spp. Ethyl acetate for culture fluid extracts and ethanol for mycelial mass were used as a negative control.

**Statistical processing methods.** To obtain reliable results, experimental studies, depending on the conditions of analysis and the requirements of mathematical planning, were performed in 3 replicates. After register the studied indicators, their reliable values were calculated by statistical methods of analysis and found the following indicators: the values of standard deviations, coefficients of variation, confidence intervals. The tables show the average statistically significant data with a 95% probability. Statistical processing of the obtained results was performed using an application program for working with spreadsheets Microsoft Office Excel 2003, 2013 (Microsoft Corporation, USA).

**Results**

The study of *F. officinalis* strains *IBK-5004, IBK-2497, IBK-2498* antimicrobial activity of culture fluid ethyl acetate extracts and mycelial mass aqueous-ethyl extracts against gram-positive *Staphylococcus aureus* (B-918), *Bacillus subtilis* (B-901) and gram-negative *Escherichia coli* (B-906), *Pseudomonas aeruginosa* (B-900), *Klebsiella pneumoniae* (M-123) bacteria by DDM was conducted.

During the experiment it was found that all studied *F. officinalis* strains under these cultivation conditions and in the presence of satisfactory growth did not show antimicrobial activity against test organisms: *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*.

Antimicrobial activity of mycelial mass and culture fluid extracts was detected against gram-positive bacteria *Staphylococcus aureus* and gram-negative bacteria *Klebsiella pneumoniae* (Table 2). In relation to *Staphylococcus aureus*, high antimicrobial activity of culture fluid extracts was detected on the 21st day of cultivation, on the 28th day it reached a maximum — the growth retardation zone was 15–24 mm (Table 2). The highest rates were found in *F. officinalis* IBK-5004 (20–24 mm) (Fig. 1b) and IBK-2498 (20–23 mm) strains.

Water-alcohol extracts of mycelial mass showed weak antimicrobial activity after 14 days of cultivation (7–8 mm). However, in strains IBK-5004 and IBK-2498 on the 21st and 28th day of cultivation the growth retardation zone for *Staphylococcus aureus* exceeded the positive control values (Table 2).

Antimicrobial activity against *Klebsiella pneumoniae* in culture fluid extracts of *F. officinalis* IBK-5004, IBK-2497, IBK-2498 strains was mani-
fested on the 21st and 28th day of cultivation. The highest antimicrobial activity against *Klebsiella pneumoniae* was found in the culture fluid of the *F. officinalis* strain IBK-5004, the diameter of the growth retardation zone was 18 mm on the 28th day of cultivation (Table 2, Fig. 1a).

In contrast to the culture fluid, the mycelial mass extracts showed moderate activity on the 14th day of cultivation (7-8 mm), the maximal activity was recorded on the 28th day (12–22 mm). The most active strain was *Fomitopsis officinalis* IBK-2498 (Fig. 2b).

Table 2: Antimicrobial activity of culture fluid ethyl acetate extracts and mycelial mass 70% water-alcohol extracts of strains from mushroom culture collection (IBK)

<table>
<thead>
<tr>
<th>Species, strain</th>
<th>Cultivation day</th>
<th>Microorganisms culture</th>
<th>Ethyl acetate extract of the culture fluid</th>
<th>Water-alcohol extract of mycelial mass</th>
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<tr>
<td>Fomitopsis officinalis, IBK-5004</td>
<td>7</td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>14</td>
<td>6.1 ± 0.2</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>21</td>
<td>20.3 ± 0.3</td>
<td>8.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>28</td>
<td>24.1 ± 0.4</td>
<td>18.1 ± 0.3</td>
<td></td>
</tr>
<tr>
<td>Control+</td>
<td></td>
<td>18 ± 0.1</td>
<td>16 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Control−</td>
<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
<tr>
<td>Fomitopsis officinalis, IBK-2497</td>
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<td>0 ± 0</td>
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<tr>
<td></td>
<td>21</td>
<td>15.2 ± 0.3</td>
<td>9.4 ± 0.3</td>
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<tr>
<td></td>
<td>28</td>
<td>19.4 ± 0.5</td>
<td>15.3 ± 0.1</td>
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<tr>
<td>Control+</td>
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<td>18.8 ± 0.1</td>
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<td>Control−</td>
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<td>Fomitopsis officinalis, IBK-2498</td>
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<td>0 ± 0</td>
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<tr>
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<td>6.1 ± 0.1</td>
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<tr>
<td>Control+</td>
<td></td>
<td>18.8 ± 0.1</td>
<td>16.7 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>Control−</td>
<td></td>
<td>0 ± 0</td>
<td>0 ± 0</td>
<td></td>
</tr>
</tbody>
</table>

Notes. "Control+" — antifungal-antibiotic solution Gentamycin sulphate. "Control−" — ethyl acetate for experiment with culture fluid, ethanol for experiment with mycelial mass. Growth retardation zone absence — the test culture is not sensitive to a given concentration of the specimen; the diameter of the growth retardation zone is less than 10 mm — the culture is moderately sensitive to a given concentration of the specimen; the diameter of the growth retardation zone is more than 10 mm — high sensitivity of the test culture to a given concentration of the specimen.
Figure 1: Antibacterial activity of ethyl acetate extract of *Fomitopsis officinalis* IBK-5004 culture fluid against: (a) *Klebsiella pneumonia* (negative control, the 14th, 21st, 28th days of cultivation); (b) antibacterial activity of ethyl acetate extract of *Fomitopsis officinalis* IBK-5004 culture fluid against *Staphylococcus aureus* (negative control, the 14th, 21st, 28th days of cultivation)

Figure 2: Antibacterial activity of ethyl acetate extract of *Fomitopsis officinalis* IBK-2498 mycelial mass against: (a) *Staphylococcus aureus* (negative control, the 14th, 21st, 28th days of cultivation); (b) antibacterial activity of ethyl acetate extracts of *Fomitopsis officinalis* IBK-2498 mycelial mass against *Klebsiella pneumonia* (negative control, the 14th, 21st, 28th days of cultivation)

Discussion

The antibiotic formation process is related to the general metabolic processes in fungal cells. Antibiotic biosynthesis occurs in the slow growth phase of the culture (the trophophase end) and reaches a maximum in the stationary growth phase. During this period, the culture fluid is enriched with metabolic products and cell autolysis products, there is an intensive biosynthesis process and maximum antibiotics accumulation. In the process of fungal culture active growth, the cells enzymatic status changes, inducers of secondary metabolism appear and induce mechanisms that inhibit cell proliferation and active growth, stressful situations, activate the antibiotic formation process.

The biological activity of many secondary metabolites which *F. officinalis* is able to synthesize has been experimentally proven [14, 15, 16, 22, 23, 26, 27]. It should be noted that polyresistant mycobacteria, which have been shown to be resistant to two major anti-tuberculosis drugs, isoniazid and rifampicin, are of particular concern. It has been experimentally proven that tender fungus extracts show high antibacterial activity against the pathogenic bacterium *Mycobacterium tuberculosis* [7, 12, 28, 29], bacteriocidal activity against *Bacillus anthracis*, *B. subtilis*, bacteriostatic activity against *Micrococcus luteus* and bacteriolytic to *Vibrio* species [12, 14–16]. High antibacterial activity of agaric acid and lanostane triterpenoids synthesized by *F. officinalis* in the process of metabolism has been established [14, 27, 30]. In the German and Swedish pharmacopoeia, agaric acid is a part of the drugs used in the treatment of patients with tuberculosis [14]. According to Airapetova et al. (2010) lipid fraction from the *F. officinalis* fruiting body has a pronounced antimicrobial effect against gram-positive cocci of the genus *Staphylococcus*: *Staphylococcus aureus* (21–25 mm), *Staphylococcus epidermidis* (22 mm), gram-negative microorganisms *Shigella* and spore-forming microorganisms of the genus *Bacillus*: *Bacillus subtilis* (17 mm), *Bacillus anthracoides* (17 mm). In our experiment, antimicrobial activity against *Bacillus subtilis* was absent. It can be assumed that the studied strains have a low level of antimicrobial substances biosynthesis (below the sensitivity of the applied detection method) and in the future it is necessary to increase the terms and to change conditions of cultivation.

Methicillin-resistant *Staphylococcus aureus* (MRSA) is a multidrug-resistant *Staphylococcus aureus* that causes nosocomial and community-acquired infections. MRSA infections today pose a serious health care problem [6, 31–34]. It is important to note that the strains studied by us synthesize biologically active substances that are able to overcome resistance to methicillin-resistant *Staphylococcus aureus* and are effective against the test bacterium *Klebsiella pneumoniae*, which is characterized by a significant variety of antibiotic resistance spectra. The variability of *Klebsiella* species resistance to existing antimicrobial drugs and the emergence of strains resistance genes dangerous for the spread confirms the need for continuous monitoring of infectious agents’ antibiotic resistance with analysis of their resistance mechanisms, as well as new producers of antimicrobial substances finding.
Conclusions

Thus, it has been found that all studied strains of *F. officinalis* (IBK–5004, IBK–2497, IBK–2498) are able to synthesize substances that inhibit the growth of individual test organisms (*Staphylococcus aureus* and *Klebsiella pneumoniae*). It was found that extracts of both culture fluid and mycelial mass show high antibacterial activity against *Staphylococcus aureus*. The studied cultures of *F. officinalis* have the potential as producers of antimicrobial substances that overcome these forms of bacteria drug resistance, i.e. those forms of resistance, the spread of which is of greatest concern to specialists.

Funding

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References


Problematics. Based on the analysis of the worldwide reports, it is evident that the antibiotic resistance is a major concern for the human health and the development of new antibiotics is a priority. The resistance of bacteria to antibiotics is a complex process that involves multiple factors, such as the resistance mechanisms of the bacteria, the selection pressure of the antibiotics, and the misuse and overuse of antibiotics. The resistance of bacteria to antibiotics is a global issue that requires international cooperation and efforts to control and prevent the spread of resistant bacteria.

Meta. The results of the study indicate that the use of natural antimicrobial agents, such as fomitopis officinalis, can be an effective tool in the fight against antibiotic resistance. The use of natural antimicrobial agents is a promising approach that could help to reduce the emergence and spread of antibiotic resistance.
Методика реализации. Проведено дослідження in vitro антимикробної активності етиловитратних екстрактів культуральної ріднин та водно-етилових екстрактів міцеліальної маси для штамів P. aeruginosa IBK-5004, IBK-2497, IBK-2498 проти грам-позитивних Staphylococcus aureus (B-918), Bacillus subtilis (B-901) та грам-негативних Escherichia coli (B-906), Pseudomonas aeruginosa (B-900), Klebsiella pneumoniae (M-123) бактерій диск-дифузійним методом.


Висновки. Встановлено, що екстракти міцеліальної маси та культуральної ріднин штамів P. aeruginosa IBK-5004, IBK-2497, IBK-2498 мають високу антимикробну активність відносно Staphylococcus aureus. Помірну антимикробну активність до Klebsiella pneumoniae спостерігали на 21-шу та 28-му добу культивування.

Ключові слова: екстракти культуральної рідини, культура міцелій, антимікробна активність.