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Antimicrobial activity of fungal extracts identified from thirty-two lichen species

The isolation of fungi from lichens, the study of their properties and the determination of their biological activity make an actual contribution to the production of a new antibiotic by the pharmaceutical industry. During the research, 22 lichen samples were collected. When determining the type of lichens, the characteristics of their growth were taken into account and the morphological identification was made according to the description of the monograph by Wang (2012). 19 fungal isolates were isolated from the core apothecia of lichen samples. Purified isolates were identified by detecting fungal DNA using PCR. These isolates were saprophytes, phytopathogens, pathogens of endolichene and symbiotic fungi. The antimicrobial properties of 19 species of identified fungi were determined against strains of *Escherichia coli* 25922 B-RKM 0447, *Staphylococcus aureus* 6538 — RKM 0470, *Serratia marcescens* B-RKM 0832 and antifungal activity against *Candida albicans* 885–653, 8 active types were identified: *Chaetomium globosum*, *Trichoderma atroviride*, *Fungal sp.*, *Coniochaeta ligniaria*, *Trichoderma harzianum*, *Hypocrea lixii*, *Daldinia loculata*, *Trichoderma viride*, *Neurospora tetraspora*. The obtained extracts against the strains *S. marcescens*, *E. coli*, *St. aureus*, *C. albicans* showed an average sensitivity of 15±0.2 – 23±0.5.

Keywords: Lichens, endolichene fungi, biological activity, identification, PCR.

Introduction

Lichens, differing in their structure, can produce a large number of secondary metabolites. This is facilitated by the fact that lichens grow slowly in nature and are difficult to propagate culturally. Cultures of mycobionts and photobionts grow faster than natural lichens; as a result, *in vitro* lichen culture is studied in order to screen biological activity and obtain a large amount of biomass to produce secondary metabolites [1-2].

Lichens synthesize secondary metabolites and most of them are produced by mycobionts aliphatic and aromatic substances, such as atranorin, parietin, usnic acid, fungal melanins. The photobiont can influence the secondary metabolism of the mycobiont [3]. In the laboratory, mycobionts can be grown without photobionts [4]. These secondary metabolites have antiviral, antibacterial, antioxidant and antitumor effects. The search for promising natural sources with such activity is an urgent problem. Secondary metabolites are easily absorbed by a living organism and are more easily biochemically broken down in the body than pharmaceutical drugs [4-5].

Endolichene fungi are distinct from mycobionts and are considered similar to endophytic fungi, but live in lichen thalli without visible signs of endophytes [5-6].

In recent studies, relatively untapped secondary metabolites other than natural substances produced by lichens have received increasing attention as interesting bioresources [7-8]. Alkaloids [9-10], quinones [11-12], terpenes [13], peptides [14] are produced from various metabolites isolated from endolichene fungi and have anticancer [15], antibacterial [16-17], antifungal [18-19], exhibits anti-inflammatory [20-21] and biological activity, possessing antioxidant properties. Due to their wide range of secondary metabolites, they can play an ecologically important role in resistance to abiotic and biotic stresses, as well as in the detoxification of secretory substances of endolichene bacteria [22].

Lichenicolous fungi are a group that has successfully developed on the visible thalli of lichens [23]. Since asymptomatic lichens have been found to contain lichen fungi [24], it is difficult from a scientific point of view to determine the difference between lichen mycobionts and endolichene fungi. Endolichene fungi consist mainly of various taxa belonging to the main genus *Ascomycota* [25].

Lichenicolous fungi represent a successfully developed group on lichen thalli visibly [23]. Being found that asymptomatic lichens harbored lichenicolous fungi, it is hard to distinguish lichenicolous fungi and endolichenic fungi scientifically. Endolichenic fungi are comprised of diverse taxa that mainly belong to major lineages of Ascomycota. It is known that the diversity and structure of the community of endolichenic fungus depend on the host of the origin of lichens, the type of photobionts, geographical location and climate [24-25].

Lichens by suppressing the oxidation of cellular macromolecules prevent mutagenesis or carcinogenesis.

The purpose of the study is to determine the type of lichen from lichens — identification by separating fungi, determining the DNA of fungi by PCR method. The biological activity of identified fungi determine.

Experimental

Materials and methods

The following methods were used to study the selected samples:

1. *Description of lichen samples, distinction of species.* Lichens are different in color, growth pattern and have three main morphological types of lichen thallus.

They are:

– squamulose (found mainly on rocky substrates and tree bark);

– foliose (found in different types of substrate);

– fruticose (found on tree branches (hanging layers) or in the soil (vertical layers). Samples of lichens in the amount of 22 pieces collected in the Komarovsky Bereg Nature Reserve, Saint-Petersburg, Russian Federation. The research was conducted in the laboratory of the Kazakh University of Technological and Business (Astana, Kazakhstan).

2. *Separation of lichens.* One fragment of the studied lichen was placed in a sterile Petri dish using a scalpel. After rinsing in distilled water, 95 % ethanol was washed for 1 min, then soaked for 3 minutes and washed in distilled water. Next, cutting off the edges of each part of the lichen fragment, plant the middle part in a finished medium of 1×1 mm. Yang et al. [26]. Incubation was carried out for 7 days in a dark place at room temperature. Morphological features were described, and then the species were identified by sequencing. The separation of fungi from lichens and their molecular genetic identification were carried out at the “National Center for Biotechnology”.

3. *Spectrophotometric determination of DNA concentration.* The method of measuring DNA concentration using a nanodrop is based on the physical principle of light absorption by materials containing DNA. The PCR reaction was performed with primers ITS5 5'-GGAAGTAAAAGTCGTAACAAGG-3' and ITS 4 5'-TCCTCCGCTTATTGATATGC-3' in a total volume of 30 µl. The PCR mixture contained 2 ng of DNA, 1 unit. The PCR program was performed using the GeneAmp PCR System 9700 (Applied Biosystems) amplifier.

Electrophoretic analysis of amplification products. Analysis of amplified target DNA fragments was carried out by separating DNA fragments in a 1.5 % agarose gel.

4. *Nucleotide sequence determination.* The sequencing reaction was performed using BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, followed by fragment separation on a 3730xl DNA Analyzer (Applied Biosystems).

Nucleotide sequences were analyzed and combined into a common sequence in the software SeqMan (DNA Star). Then the terminal fragments (nucleotide sequences of primers, fragments with a low quality score) were removed. The obtained sequences were identified in GeneBank using the BLAST algorithm. The results are shown in Table 1.

Taking into account the literature data indicating the presence of nucleotide sequences in international banks GeneBank (<http://www.ncbi.nlm.nih.gov/>), Ribosomal Database Project (RDP-II) (<http://rdp.cme.msu.edu/html/>). The Muscle algorithm was used to align nucleotide sequences; trees were constructed using the nearest neighbor joining method (Neighbor-Joining NJ).

5. *Propagation in liquid medium.* Micromycetes grown on Chapeka dextrose agar medium were incubated with micromycete fragment in Chapeka dextrose broth liquid medium. Incubation was carried out at room temperature in the dark for 7 days.

6. *Cultivation on solid medium, fermentation.* Micromycetes grown in the liquid medium of Czapek dextrose broth were transferred to a solid medium. All mushrooms were grown in the dark at room

temperature for 25 days. Extraction with ethyl acetate separated the oily mixtures from light yellow to brick red in color. The antimicrobial activity of the separated mixtures was studied.

7. *Determination of biological activity.* The liquid of mushrooms isolated from lichens was fermented in small quantities and an extract was obtained. The antimicrobial properties of fungi were determined by the agar diffusion method. Pits with a diameter of 8 mm were made into the thickness of the plate and test microbes were inoculated onto the agar surface. A day later, fractional solutions of the tested products were injected into the cells. The growth area of the test culture studied by monosporic strains of the fungus was estimated in diameter for 1 day (for bacteria) and for 2 days (for fungi). The results obtained were explained as follows: 0 — lack of activity, up to 12 mm—poor sensitivity; from 13 to 29 mm — medium sensitivity, 30 mm and high sensitivity. Extracted extracts *S. marcescens*, *E.coli*, *St. aureus* on microorganisms antifungal effect of *C. albicans*.

Statistical information

The data obtained were mathematically processed using Microsoft Excel 2019, as well as using regression analysis and Spearman correlation coefficient.

Results and Discussion

During the study, we collected samples of 22 species of lichens. Fungal isolates of the phylum Ascomycota were isolated from lichens. Figure 1 shows some of the lichen specimens.











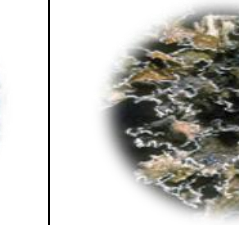
			
<i>Hypogymnia physodes</i>	<i>Xanthotia parietina</i>	<i>Peltigera protextata</i>	<i>Physcia adscendens</i>
			
<i>Cladonia furcata</i>	<i>Chaenotheca furfuracea</i>	<i>Vulpicida pinastri</i>	<i>Cladonia coniocraea</i>
			
<i>Melanohalea exasperata</i>	<i>Cladonia pityrea</i>	<i>Hypogymnia tubulosa</i>	<i>Tuckermannopsis chlorophylla</i>

Figure 1. Species of lichens collected in the Komarovsky nature Reserve, Saint-Petersburg, Russia

To isolate fungi from lichens, ready-made Chapeka dextrose agar was used. Some of the growing fungal colonies were transplanted to a new medium. As a result of three repeated inoculations, micromycetes isolated from lichens were introduced into a liquid medium of Czapek dextrose broth with a micromycete fragment. Incubation was carried out at room temperature in the dark for 7 days (Fig. 2).

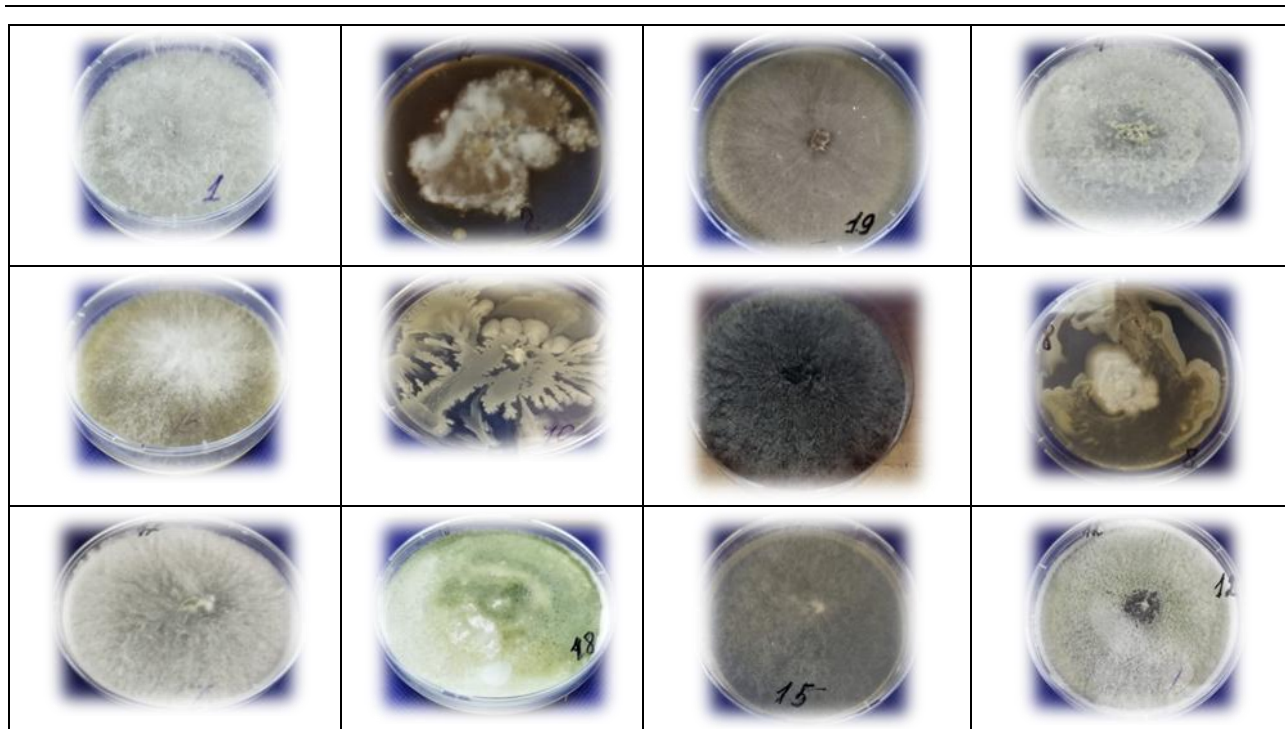


Figure 2. Fungi isolated from lichens

Isolation of DNA from fungal strains

Incubation was carried out at room temperature in the dark for 7 days. Morphological features were described, and then species identification was carried out using sequencing (Table 1).

Table 1

Identification of results using nucleotide sequence analysis

N samples	Sequence	Organism	Personality, %
1	ITS5 5'-GGAAGTAAAA GTCGTAACAAGG-3' and ITS 4 5'-TCCTCCGCTTATT GATATGC-3'	<i>Desmazierella acicola</i>	97.7
2		<i>Chaetomium globosum</i>	98.2
3		<i>Coniochaeta hoffmannii</i>	99.0
4		<i>Trichoderma atroviride</i>	100.0
5		<i>uncultured Madurella</i>	90.5
6		<i>Fimetariella rabenhorstii</i>	92.3
7		<i>Fimetariella rabenhorstii</i>	92.3
8		<i>Fungal sp.</i>	96.4
9		<i>Coniochaeta luteorubra</i>	95.5
10		<i>Hypocrea lixii</i>	96.3
11		<i>Daldinia loculata</i>	98.5
12		<i>Umbelopsis sp.</i>	87.9
13		<i>Nigrospora oryzae</i>	98.0
14		<i>Trichoderma harzianum</i>	99.1
15		<i>Geopyxis carbonaria</i>	98.6
16		<i>Mortierella turficola</i>	98.7
17		<i>Trichoderma koningiopsis</i>	98.9
18		<i>Nigrospora oryzae</i>	98.6
19		<i>Trichoderma viride</i>	100.0
20		<i>Gelasinospora tetrasperma</i>	94.0
21		<i>Neurospora tetraspora</i>	100.0
22		<i>Coniochaeta ligniaria</i>	89.4

As shown in the Table 1, 19 fungal strains out of 22 fungi were identified. To check the activity of isolated strains, *E. coli*, *St. aureus*, *S. marcescens*, *C. albicans* strains were used. Antimicrobial and antifungal activity was tested by disco-diffusion agar method (Table 2).

Table 2

Antimicrobial and antifungal activity

N	Name of the mushroom	Antimicrobial effect			Antifungal effect
		<i>E. coli</i>	<i>St. aureus</i>	<i>S. marcescens</i>	<i>C. albicans</i>
1	<i>Desmazierella acicola</i>	15 ± 0.1	14 ± 0.21	16 ± 0.09	14 ± 0.09
2	<i>Chaetomium globosum</i>	17 ± 0.2	18 ± 0.5	18 ± 0.33	19 ± 0.24
3	<i>Coniochaeta hoffmannii</i>	13 ± 0.1	12 ± 0.2	13 ± 0.5	11 ± 0.5
4	<i>Trichoderma atroviride</i>	21 ± 0.3	19 ± 0.22	21 ± 0.5	19 ± 0.5
5	<i>Uncultured madurella</i>	12 ± 0.5	12 ± 0.3	11 ± 0.5	10 ± 0.09
6	<i>Fimetariella rabenhorstii</i>	12 ± 0.5	12 ± 0.7	11 ± 0.8	10 ± 0.5
7	<i>Fungal sp.</i>	19 ± 0.5	20 ± 0.4	20 ± 0.5	18.8 ± 0.5
8	<i>Coniochaeta luteorubra</i>	15 ± 0.5	13 ± 0.5	15 ± 0.5	15 ± 0.5
9	<i>Hypocrea lixii</i>	22 ± 0.5	21 ± 0.5	23 ± 0.5	20 ± 0.5
10	<i>Daldinia loculata</i>	20 ± 0.5	20 ± 0.5	22 ± 0.4	21 ± 0.5
11	<i>Umbelopsis sp.</i>	15 ± 0.5	15 ± 0.3	15 ± 0.5	15 ± 0.5
12	<i>Nigrospora oryzae</i>	14 ± 0.8	13 ± 0.5	14 ± 0.2	14 ± 0.25
13	<i>Trichoderma harzianum</i>	22 ± 0.5	22.6 ± 0.5	23 ± 0.5	21 ± 0.5
14	<i>Geopyxis carbonaria</i>	15 ± 0.6	15 ± 0.5	17 ± 0.8	14 ± 0.5
15	<i>Mortierella turficola</i>	21 ± 0.5	21 ± 0.5	22 ± 0.5	20 ± 0.9
16	<i>Trichoderma koningiopsis</i>	22 ± 0.1	20 ± 0.5	19 ± 0.45	18 ± 0.4
17	<i>Trichoderma viride</i>	19 ± 0.2	19 ± 0.5	21 ± 0.38	19 ± 0.5
18	<i>Gelasinospora tetrasperma</i>	15 ± 0.5	15 ± 0.33	17 ± 0.5	16 ± 0.3
19	<i>Neurospora tetraspora</i>	20 ± 0.5	19 ± 0.5	21 ± 0.5	22 ± 0.5

As a result, the antimicrobial effect of *Chaetomium globosum*, *Trichoderma atroviride*, *Fungal sp.*, *Trichoderma harzianum*, *Hypocrea lixii*, *Daldinia loculata*, *Trichoderma viride*, *Neurospora Tetraspora* showed an average sensitivity in the range of 15±0.2 – 23±0.5.

Conclusion

Among lichens with antimicrobial and antifungal properties, 19 species of fungi have been identified and 8 active types have been recorded: *Chaetomium globosum*, *Trichoderma atroviride*, *Fungal sp.*, *Coniochaeta ligniaria*, *Trichoderma harzianum*, *Hypocrea lixii*, *Daldinia loculata*, *Trichoderma viride*, *Neurospora tetraspora*.

Extracts obtained from *S. marcescens*, *E. coli*, *St. aureus*. The average sensitivity of *C. albicans* was 15 ± 0.2 – 23 ± 0.5. In the future, it is planned to isolate pure antibiotics from them and carry out their structural identification.

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Қыналардың отыз екі түрінен оқшауланған саңырауқұлақ сығындыларының микробқа қарсы белсенділігі

Саңырауқұлақтарды қыналардан бөлу, қасиеттерін зерттеу және олардың биологиялық белсенділігін анықтау фармацевтика өнеркәсібінің жаңа антибиотик өндірісіне қосқан нақты үлесі. Зерттеу барысында қыналардың 22 үлгісі жиналды. Қыналардың түрін анықтауда олардың өсу ерекшеліктері ескеріліп, морфологиялық сәйкестендіру Wang (2012) монографиясындағы сипаттамасына сәйкес жүргізілді. Қына үлгілерінің апотецияларынан 19 саңырауқұлақ изоляты оқшауланды. Тазартылған изоляттар ПТР көмегімен саңырауқұлақтардың ДНҚ-н анықтау арқылы айқындалды. Бұл изоляттар сапрофиттер, фитопатогендер, эндолихен қоздырғыштары және симбиотикалық саңырауқұлақтар болды. Анықталған саңырауқұлақтардың 19 түрінің микробқа қарсы қасиеттері *Escherichia coli* 25922 В-РКМ 0447, *Staphylococcus aureus* 6538 — РКМ 0470, *Serratia marcescens* В-РКМ 0832 штамдарында және *Candida albicans* 885–653 саңырауқұлаққа қарсы белсенділікке тексеріліп, *Chaetomium globosum*, *Trichoderma atroviride*, *Fungal sp.*, *Coniochaeta ligniaria*, *Trichoderma harzianum*, *Hypocrea lixii*, *Daldinia loculata*, *Trichoderma viride*, *Neurospora tetraspora* сияқты 8 белсенді түрлері анықталды. Алынған сығындылар *S. marcescens*, *E. coli*, *St. aureus*, *C. albicans* штамдарына қарсы орташа сезімталдықты $15\pm 0,2 - 23\pm 0,5$ аралығында көрсетті.

Клт сөздер: қыналар, эндолихенді саңырауқұлақтар, биологиялық белсенділік, идентификациялау, ПТР.

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Антимикробная активность экстрактов грибов, выделенных из тридцати двух видов лишайников

Выделение грибов из лишайников, изучение их свойств и определение их биологической активности являются реальным вкладом фармацевтической промышленности в производство нового антибиотика. В ходе исследования было собрано 22 образца лишайников. При определении типа лишайников учитывались особенности их роста, а морфологическая идентификация проводилась в соответствии с описанием в монографии Wang (2012). 19 изолятов грибов были выделены из сердцевинных апотеций образцов лишайников. Очищенные изоляты были идентифицированы путем определения ДНК грибов с помощью ПЦР. Эти изоляты были сапрофитами, фитопатогенами, возбудителями эндолихена и симбиотическими грибами. Антимикробные свойства 19 видов идентифицированных грибов были определены в отношении штаммов *Escherichia coli* 25922 В-РКМ 0447, *Staphylococcus aureus* 6538 — РКМ 0470, *Serratia marcescens* В-РКМ 0832 и противогрибковой активности в отношении *Candida albicans* 885–653, было идентифицировано 9 активных видов: *Chaetomium globosum*, *Trichoderma atroviride*, *Fungal sp.*, *Coniochaeta ligniaria*, *Trichoderma harzianum*, *Hypocrea lixii*, *Daldinia loculata*, *Trichoderma viride*, *Neurospora tetraspora*. Полученные экстракты против штаммов *S. marcescens*, *E. coli*, *St. aureus*, *C. albicans* показали среднюю чувствительность в пределах $15\pm 0,2 - 23\pm 0,5$.

Ключевые слова: лишайники, эндолихеновые грибы, биологическая активность, идентификация, ПЦР.

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