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STUDY ON ANTIMICROBIAL ACTIVITY OF VARIOUS EXTRACTS OF SAXICOLOUS LICHEN *LECANORA MURALIS*

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Antimicrobial activity of aqueous, acetone, methanol, and ethanol extracts of saxicolous lichen *Lecanora muralis* sampled from Odzun, Lori province of Armenia were studied. Aqueous extract was not demonstrated any significant antibacterial activity neither Gram positive nor Gram negative bacteria. All other extracts exhibited antimicrobial activity only against only tested Gram-positive bacteria. The minimal inhibitory concentration (MIC) value of acetone extract was 57 μg/μl against *Bacillus subtilis*.

Saxicolous lichen – Lecanora muralis – extract – antimicrobial activity

Ուսումնասիրվել է Օձունից (Լոռու մարզ, ՀՀ) հավաքված էպիլիթային քարաքոս *Lecanora muralis*-ի ջրային, մեթանոլային, էթանոլային և ացետոնային լուծամզվածքների հակաբակտերիական ակտիվությունը: Ջրային լուծամզվածքը չի դրսևորել նշանակալի հակաբակտերիական ակտիվություն թե՛ գրամբացասական և թե՛ գրամդրական բակտերիաների նկատմամբ: Մյուս լուծամզվածքները դրսևորել են հակաբակտերիական ակտիվություն միայն տեստավորված գրամդրական բակտերիաների նկատմամբ: Ացետոնային լուծամզվածքի նվազագույն արգելակիչ կոնցենտրացիան *Bacillus subtilis*-ի նկատմամբ կազմել է 57 մկգ/մկլ:

Էպիլիթային քարաքոս – Lecanora muralis – լուծամզվածքներ – հակաբակտերիական ակտիվություն

Изучена антибактериальная активность водных, метанольных, этанольных и ацетоновых экстрактов эпилитного лишайника *Lecanora muralis*, собранного в Одзуне, Лоринской области Армении. Водный экстракт лишайника не показал активность против не грамположительных, и не грамотрицательных бактерий. Метанольные, этанольные и ацетоновые экстракты талома лишайника демонстрировали антибактериальную активность только против протестированных грамположительных бактерий. Минимальная ингибирующая активность (МИК) против *Bacillus subtilis* ацетонового экстракта лишайника составляла 57 мкг/мкл.

Эпилитный лишайник – Lecanora muralis – экстракты – антимикробаная активность

Introduction

Lichens are complex symbiotic communities of fungal (mycobiont) and algae (photobiont) components. The mycobiont is mainly represented by *Ascomycota* (98%), and the rest by *Basidiomycota* and other fungi. The most common photobionts are *Trentepohlia*, *Trebouxia* (green algae) և *Nostoc* (cyanobacteria) [8]. There are various

types of lichens based on their habitat: epilithic or saxicolous (lives on rocks), epiphytic (on tree bark), epixil (on rotten wood), epiphilic (on needles), epigey (on soil), epibriophil (on moss), ambiophilic (in water). Recent studies have shown that bacteria are also an integral part of lichens, the degree of this symbiotic relationship depends on the type of lichen [12]. Due to this multi-component relationship, lichens have adapted to living in extreme environments, such as high-temperature, dehydrated environments in Antarctica, humid rainforests, and deserts [7].

Lichens have come to the attention of scientists due to their great biotechnological potential, especially due to the production of various biologically active compounds. Lichens, particularly epiphytic lichens, produce unique secondary products that have been used in traditional medicine as analgesics, antipyretics, and anti-inflammatory agents. The use of various extracts of these organisms in pharmacy to produce a new generation of antibacterial, antifungal, antiviral, cytotoxic, antioxidant preparations is gaining momentum [15].

Few studies aimed to evaluate the biotechnological potential of saxicolous lichens. Thus, recently the antioxidant and antibacterial activities of three saxicolous lichens *Cladonia furcata, Lecanora atra* and *Lecanora muralis* were studied. It was shown high antioxidant and antibacterial activities of representatives of the genus *Lecanora.* High cytotoxic activity of studied saxicolous lichens on FemX and LS 174 cell lines has been revealed as well [9]. Hence the extracts in the preceding study were prepared using acetone, this may limit the evaluation of larger spectrum of secondary metabolites produced by lichens, as some studies show that methanolic extracts exhibit highest biological activity [11,9,2]

Saxicolous lichens occur on hardened basalt, pumice, rhyolite, granite, sandstones, and limestones at altitudes of 20-2800 m and described in Europe, Asia, North America, South America, Africa, Macaroni, Oceania, Australia, and Sonora. Some species contain isosunic acid instead of usnic acid [8].

The lichen flora of Armenia is rich in the region, even with endemic lichens, the species composition and biotechnological potential of which have not been properly studied [13]. The study aimed to evaluate the biological activity and biotechnological potential of lichens distributed in Armenia, were initiated recently [13, 3]. Based on the foregoing, the main purpose of this study was to study the antibacterial activity of the saxicolous lichen *Lecanora muralis* sampled in Armenia.

Materials and methods. Object of study. ***Object of study***. The object of study in this work was the samples of saxicolous lichen thalli collected from the surroundings of Odzun village of Lori province of the Republic of Armenia (1500 m above sea level). The sampling was carried out on July-August 2019. The sampling site was chosen due to high humidity and high altitude promoting rapid growth of lichens. The sampled lichens were taken to laboratory (Department of Biochemistry, Microbiology and Biotechnology, YSU), where they were dried, cleaned of soil debris, other elements, identified and prepared in the herbarium. The sampled lichen was identified according to standard methods [10], considering the morphological characteristics. According to the identification key it was identified as a species belonging to the genus *Lecanora - Lecanora muralis*.

The following strains of gram-positive and gram-negative bacteria stored in the collection of bacterial cultures of YSU, Department of Biochemistry, Microbiology, and Biotechnology were used as test organisms: *Bacillus subtilis* A1*, Staphylococcus aureus* 5233*, Salmonella typhimurium* MDC1754 and *Escherichia coli M*17.

Lichen extracts. The lichen thalli (10 g) were milled with an electric grinder. To obtain aqueous extract, the grinded lichens were dissolved in distilled boiled water, then left for extraction for 24 *h*. The resulting water tinctures were filtered through 0.22 sterile millipore filters and dried under aseptic conditions to avoid environmental contamination. A solvent (methanol,

ethanol, acetone) in the ratio of 1:5 was added to the powder mass of lichens, then extracted by stirring with a magnetic stirrer for 24 hours in dark conditions at room temperature to obtain the extracts. The suspension to separate the extract from the biomass was centrifuged at 15 ° C at 4,000 rpm. To evaporate the solvent from the obtained extracts, they were placed in a vacuum evaporator until a dry mass was obtained. All dry extracts were weighted and stored at –18°C. The resulting mass was weighed, then dissolved in dimethyl sulfoxide (DMSO) (Sigma-Aldrich) and stored at -4°C. For experiments, the final concentration of DMSO in extracts was 5%.

Antibacterial activity of lichen extracts. The antibacterial activity of lichen extracts was determined by diffusion assay by incubation of bacterial strains on solidified Mueller-Hinton broth (MHB) [6]. Disk diffusion and the minimum inhibitory concentration by agar dilution methods [6]. Filter paper (Whatman) disks (5 mm in diameter) were sterilized at 121°C by autoclaving for 21 minutes. Overnight cultures were obtained for lawn growth of test organisms, inoculums were made using the overnight cultures. Then, pre-impregnated by extracts paper disks were placed on the surface of the agar layer at an equal distance of 1.5-2 cm from the edges of the plate, then incubated at 24 h at 37 °C. Filter papers soaked in 5% sterilized DMSO served as a negative control. The experiments were performed by three replicates, and the diameter of the inhibition zones (IZ) around the disks was taken as a unit of measure of the antibacterial activity.

Minimal inhibitory concentration (MIC). To determine the MIC, serial dilutions of lichen acetone extract with sterilized distilled water were performed [6]. The resulting dilute solutions were dripped onto pre-sterilized paper discs and placed on the surface of an inoculated medium. After 24 h of incubation, the minimum inhibitory concentration was determined by measuring the growth inhibition bands formed around the discs. The values obtained were the average data of experiments performed at least three time.

Results and discussion

In this study, we targeted to evaluate the antibacterial activity of *Lecanora muralis* sampled in Armenia. The thallus of *L. muralis* is a placoid 1.5 – 3.5 cm in diameter, more often it is 0.5-2 mm thick and even thicker in the central part. It often forms small rosette-like formations and is firmly attached to the stone (Fig. 1). The prethallus is absent or vestigial, often dark green or gray from the edges, 0.5-1 mm wide, with irregular branching.

Fig. 1. Saxicolous lichen *Lecanora muralis* thalli on the rock at the sampling site.

The antibacterial activity of lichen extract was tested against both gram-positive and gram-negative bacteria. The antibacterial activities of various extracts of saxicolous lichen *L. muralis* are presented in Table 1. The aqueous extracts did not exhibit any activity in disc diffusion assays. The lack of inhibition of the microbial growth by the aqueous extract of *L. muralis* in this study corresponds to data described earlier by other researchers [14]. However, there are also contradictory data showing that aqueous extract of some *Umbilicaria*, *Xanthoria* and *Xanthoparmelia* species have antibacterial activity against *E. coli*, *B. subtilis* and *S. aureus* [4]. Presumably, the chemicals with

antibacterial activities incorporated with lichen thallus were not soluble or were poorly soluble in water [14].

In contrast to aqueous extracts, other extracts displayed a relatively high antibacterial activity. Methanol, ethanol, and acetone extracts showed activity against gram-positive bacteria i.e., *B. subtilis* and *S. aureus*, whereas they did not show any activity against gram-negative bacteria. The highest activity was observed in case of acetone extracts. As can be seen from the table, the acetone extract of *L. muralis* exhibits growth inhibitory activity against *B. subtilis* (1.7 cm, Ø) and *S. aureus* (0.7 cm, Ø), while for the methanol and ethanol extracts bacterial growth inhibition zones were 1.1 cm and 1.55 cm for *B. subtilis,* respectively. Ethanol extract did not show any activity against *S. aureus*, while methanol extracts inhibited growth of cells (Fig 2). Presumably, acetone was the most efficient solvent for the extraction of phenolic and flavonoid compounds responsible for antibacterial activities [8].

Table 1. Antibacterial activity of L. muralis by disk diffusion assay

Test bacteria	Crude extracts, IZ* (Ø, cm)				
	Methanol	Ethanol	Acetone	Water	NG**
B. subtilis	≥ 1,1	≥ 1,5	≥ 1,7	-***	-
S. aureus	≥ 0,5	-	≥ 0,7	-	-
E. coli	-	-	-	-	-
S. typymurium	-	-	-	-	-

IZ, zone of inhibition; **NC, negative control (DMSO), *(–), no zone of inhibition*

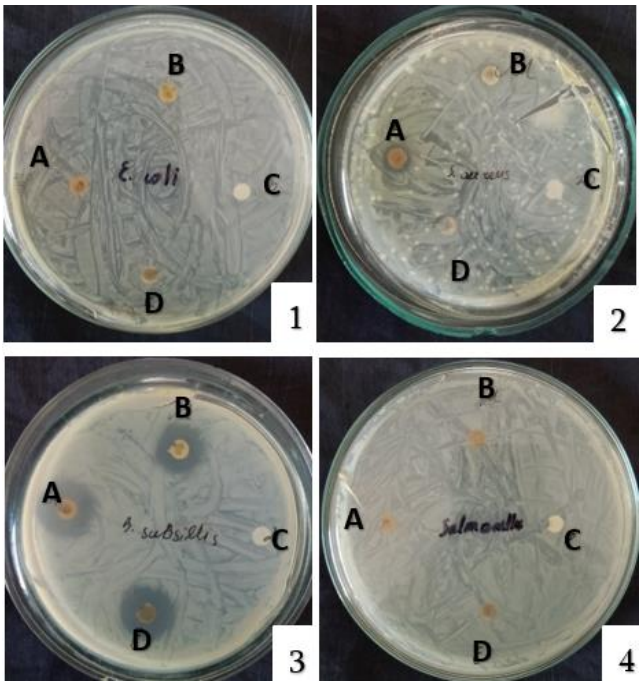

Fig. 2. Inhibition zones of different lichen extracts.
1. *E. coli*; 2. *S. aureus*; 3. *B. subtilis*; 4. *S. typhymurium*;
Discs soaked by Extracts of: A. Acetone; B. Methanol;
C. Negative control (DMSO); D. Ethanol.

Thus, the extracts exhibited a narrow spectrum of antibacterial activity with a bactericidal effect only on gram-positive bacteria. Their effect on gram-positive bacteria is probably due to the absence of an outer membrane, which makes them more sensitive to the active substances. It can be assumed that the effect of lichen extracts studied only on gram-positive bacteria is probably due to their effect on the murein-based cell wall [17].

The inhibition zone was 1.4 cm for *B. subtilis* and 1.6 cm for *S. aureus*, whether for the methanol extract 1.4 cm and 1.3 cm respectively. For the *Lecanora atra* lichen, the inhibition zone of acetone extract was 1.4 cm for *B. subtilis* and 1.6 cm for *S. aureus*. In the case of methanolic extract, it was 1.4 cm and 1.3 cm, respectively [14].

Considering that the highest antibacterial activity against gram-positive bacteria is exhibited by acetone extract, the MIC was determined by the effect of that extract on B. subtilis. To study the antibacterial activity extracts of different densities were obtained by diluting the extract, then soaking it onto pre-sterilized paper discs and placing it on the surface of an inoculated medium. After 24 hours of incubation, the MIC was calculated based on growth inhibition zones around the discs. It was ≥ 57 μg/μl.

From the data obtained, it can be stated that the most effective solvent was acetone as possibly it dissolves the most biologically active substances from the lichen thallus. The fact that *B. subtilis* is an endospore-producing bacterium suggests a possible sporicidal or sporostatic effect of the extracts, which requires further investigation.

Conclusions

From the data obtained during the studies, it can be stated that the methanol, ethanol, and acetone extracts from lichen *Lecanora muralis* thallus exhibit a narrow spectrum of antimicrobial activity on the tested Gram-positive bacteria. In virtue of these results, saxicolous lichens belonging to genus *Lecanora* should be considered as organisms with high biotechnological potential, which was proved before by various authors, but has been reported for the first time for *Lecanora* species distributed on the territory of Armenia.

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