

SCREENING OF ANTIMICROBIAL ACTIVITY OF SOME LICHEN SPECIES IN VITRO

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ABSTRACT. Antimicrobial activity of the acetone-, methanol- and aqueous- extracts of the lichens *Lecanora atra*, *L. muralis*, *Parmelia saxatilis*, *P. sulcata* and *Parmeliopsis ambigua* was explored in vitro against to six species of bacteria and 10 species of fungi, by the disc-diffusion method and determination of the minimal inhibitory concentration (MIC) by the Broth tube Dilution method. The aqueous extracts of the tested lichens didn't show any antimicrobial activity on any of the test microorganisms, whereas the acetone and methanol extracts showed an activity related to the tested organisms. The bacteria were very sensitive related to the tested fungi. The strongest antimicrobial activity was found in the acetone extract of the lichen *Parmelia sulcata* where the least measured MIC value was 0.78 mg/ml. Generally, among the bacteria the most sensitive was the species *Bacillus mycoides*, and among the fungi *Botrytis cinerea* and *Candida albicans*. The bacterium *Escherichia coli* was resistant to all extracts of the explored lichens. Generally, all explored lichens had a relatively strong antimicrobial activity, which can be very important in treatment of numerous diseases caused by tested and similar microorganisms.

KEY WORDS: Antimicrobial activity; Lichen extracts

INTRODUCTION

Lichens are symbiotic organisms consisting of fungi and a photosynthetic partner, that can be an alga or a cyanobacterium (AHMADIJAN, 1993). They usually grow on rocks, non-fertile ground, as well as epiphytes on the trees and leaves (TAYLOR *et al.*, 1995).

Lichens synthesize various bioactive components, which sometimes constitute more than 30% of the dry mass of talus (GALUN, 1988). Although there are about 20,000 species of them around the world, and even they make 8% of the terrestrial ecosystems, their biological activity and biological components are not distinguished very much (TOMA *et al.*, 2001). Various biological activities of some lichens and their components are known, such as: antiviral, anti-tumor, anti-inflammatory, analgetic, antipyretic, antiproliferative, antiprotozoal (LAWREY, 1986; HALAMA *et al.*, 2004; HUNECK, 1999). Besides, many species are used for

human nutrition, animal nutrition, for getting colors, perfumes, alcohol and in the medicine industry. (KIRMIZIGÜL *et al.*, 2003). Lichens have also, for hundreds of years, been used in many European countries as a cure for stomach diseases, diabetes, cough, pulmonar tuberculosis, wounds curing, dermatological diseases (BAYTOP, 1999; HUNECK, 1999). The usage of some lichens in the traditional medicine for many years was later justified by numerous researches which proved their antimicrobial activity (CANSARAN *et al.*, 2006; CHOUDHARY *et al.*, 2005; GULLUCE *et al.*, 2006; RANKOVIĆ *et al.*, 2007a). So, the aim of this work is to investigate the antimicrobial activity of the acetone, methanol and aqueous extract of the lichens in relation to the chosen test microorganisms, among which are the causes of the diseases of humans, animals and plants.

MATERIALS AND METHODS

Lichen samples

Samples of the lichens of *Lecanora atra* (Hudson) Ach., *L. muralis* (Schreber) Rabenh., *Parmelia saxatilis* (L.) Ach., *P. sulcata* (Taylor) and *Parmeliopsis ambigua* (Wulf.) Nyl., were collected on Borač, Serbia, in August of 2008. The demonstration samples are preserved in facilities of the Department of Biology and Ecology of Kragujevac Faculty of Science. Determination of the investigated lichens was accomplished using standard keys (PURVIS *et al.*, 1992; WIRTH, 1995; DOBSON, 2000).

Microorganisms and media

The bacteria used as test organisms in this study were as follows: *Bacillus mycoides* (IPH), *B. subtilis* (IPH), and *Staphylococcus aureus* (IPH) (Gram-positive bacteria); and *Enterobacter cloacae* (IPH), *Escherichia coli* (IPH), and *Klebsiella pneumoniae* (IPH), (Gram-negative bacteria). All of the bacteria used were isolates of the Institute for Protection of Health in Kragujevac (IPH) and the Faculty of Agriculture in Belgrade (FAB). Their identification was confirmed in the Microbiological Laboratory of Kragujevac University's Department of Biology. The fungi used as test organisms were: *Aspergillus flavus* (ATCC 9170), *A. fumigatus* (DBFS 310), *Botrytis cinerea* (DBFS 133), *Candida albicans* (IPH 1316), *Fusarium oxysporum* (DBFS 292), *Mucor mucedo* (ATCC 52568), *Paecilomyces variotii* (ATCC 22319), *Penicillium purpurascens* (DBFS 418), *P. verrucosum* (DBFS 262), and *Trichoderma harsianum* (DBFS 379). They were from the mycological collection maintained by the Mycological Laboratory within the Department of Biology of Kragujevac University's Faculty of Science (DBFS). Bacterial cultures were maintained on Müller-Hinton agar substrates (Torlak, Belgrade). Fungal cultures were maintained on potato dextrose agar and Sabourad dextrose agar (Torlak, Belgrade). All cultures were stored at 4°C and subcultured every 15 days.

Preparation of the lichen extracts

Finely pulverised thalli of the investigated lichens (50 g) were extracted using acetone, methanol and water in a Soxhlet extractor. The extracts were filtered and then concentrated under reduced pressure in a rotary evaporator. The dry extracts were stored at -18°C until they were used in the tests. The extracts were dissolved in dimethyl sulphoxide (DMSO). The final concentration for the DMSO didn't extend 2%.

Antimicrobial assays

The sensitivity of microorganisms to acetone, methanol and aqueous extracts of the investigated species of lichens was tested by measuring the inhibition zone of a given extract concentration by the disc diffusion method and by determining the minimal inhibitory concentration (MIC).

Bacterial inoculi were obtained from bacterial cultures incubated for 24 h at 37°C on Müller-Hinton agar substrate and brought up by dilution according to the 0.5 McFarland standard to approximately 10^8 CFU/ml. The suspensions of fungal spores were prepared from fresh mature (3- to 7-day-old) cultures that grew at 30°C on a PDA substrate. Spores were rinsed with sterile distilled water, used to determine turbidity spectrophotometrically at 530 nm, and then further diluted to approximately 10^6 CFU/ml according to the procedure recommended by the NCCLS (National Committee for Clinical Laboratory Standards M 38 - P, 1998).

A standard disc-diffusion method (NCCLS, M2-A5, 1993) was used to study antimicrobial activity. Müller-Hinton agar (for bacteria) or in Sabourad dextrose agar (for fungi) was seeded with the appropriate inoculum. Paper discs (7 mm diameter) were laid on the inoculated substrate after being soaked with 15 µL of lichen extract (50 mg/mL). Antimicrobial activity was determined by measuring the diameter of the inhibition zone around the disc. Streptomycin (for bacteria) and ketoconazole (for fungi) were used as controls. A DMSO solution was used as a negative control for the solvents influence. All experiments were performed in triplicate.

The minimal inhibitory concentration (MIC) was determined by the broth tube dilution method. A series of dilutions with concentrations ranging from 50 to 0.0037 mg/mL was used in the experiment for each extract against every microorganism tested. The starting solutions of extracts with a concentration of 50 mg/mL were obtained by measuring off a certain quantity of extract and dissolving it in DMSO. Two-fold dilutions of extracts were prepared in Müller-Hinton broth for bacterial cultures and Sabourad dextrose broth for fungal cultures in test tubes. The minimal inhibitory concentration was determined by establishing the visible growth of the microorganisms. The boundary dilution without any visible growth was defined as the minimal inhibitory concentration (MIC) for the tested microorganism at the given lichen extract concentration. As a positive control of growth inhibition, streptomycin was used in the case of bacteria, ketoconazole in the case of fungi. All experiments were performed in triplicate.

RESULTS

The antimicrobial activity of the tested lichen extracts against the tested microorganisms was shown in the tables, for the disc-diffusion method (Table 1) and the minimal inhibitory concentration (Table 2).

Disc-difusional method

The acetone and methanol extracts of the tested lichens showed a strong antimicrobial activity. The extracts of the lichen *Lecanora atra* inhibited five out of six tested bacteria. The greatest sensitivity to the tested species was shown by the *Bacillus mycoides* in which the largest inhibition zone were measured (22 cm for the acetone and 25 mm for the methanol extract). The extracts of the lichen *Lecanora atra* also showed an antifungal activity in relation to the all tested fungi. The zones of inhibition for the acetone and the methanol extracts were within the range of 11 to 21 mm.

The lichen *Lecanora muralis* showed a relatively strong antibacterial activity.

The largest zone of inhibition (26 mm) was measured in the methanol extract relative to the *Bacillus mycoides*. The extracts of this lichen showed a weak antifungal activity. The acetone extract inhibited two and the methanol extract seven out of ten tested fungi. The zone of inhibition for the acetone and methanol extracts were within the range 12-16 mm.

The acetone and methanol extracts of the lichen *Parmelia saxatilis* showed a very strong inhibitory influence on the tested bacteria. Larger inhibition zones were noticed in the

methanol extract, especially in relation to the *Bacillus mycoides* (24 mm). The inhibition zones for both extracts in relation to the tested fungi were 13-28 mm.

The extracts of the lichen *Parmelia sulcata* showed the strongest antibacterial activity. The acetone extract showed a stronger antibacterial effect compared to the methanol. The inhibition zones relative to the bacteria were large. They were within the range 16-28 mm for the acetone and 13-26 mm for the methanol extract. The lichen *Parmelia sulcata* had a strong antifungal activity. The measured inhibition zones in relation to the fungi were also large.

The acetone and methanol extracts of the lichen *Parmeliopsis ambigua* showed a relatively strong antimicrobial activity. The inhibition zones for both extracts in relation to the bacteria and fungi were relatively large (11-24 mm).

Minimal inhibitory concentration (MIC)

The MIC for the different extracts related to the tested bacteria and fungi were within the range 0.78-25 mg/ml. The biggest antibacterial activity was in the extracts of the lichen *Pamela sulcata*, particularly in the acetone extract, which inhibited the tested bacteria in a very low concentration (0.78 mg/mL). The lichen *P. sulcata* had a very strong antifungal activity as well. The measured MIC values related to the tested fungi were relatively low (1.56-12.5 mg/mL). The extracts of the lichens *Lecanora atra*, *Parmelia saxatilis* and *Parmeliopsis ambigua* showed relatively equal antimicrobial activity, although it should be stressed that the methanol extracts had shown a stronger inhibitory influence than the acetone. The lichen *Lecanora muralis* showed a relatively strong antibacterial effect but the antifungal effect was weak. The MIC for the acetone and methanol extracts of the lichen *L. muralis* were within the range 1.56-3.12 mg/mL in relation to the bacteria and 12.5-25 mg/mL in relation to the fungi.

DISCUSSION

The tested lichen extracts showed a relatively strong antimicrobial activity. The intensity of the antimicrobial effect of the tested extracts depended on the sort of the extract, its concentration and the tested microorganism. The aqueous extracts of the tested lichens didn't show any antimicrobial activity. That's probably because the active components produced by lichens can't be diluted or can be little diluted in water (KINOSHITA *et al.*, 1994). The antibacterial effect is stronger compared to the antifungal. These results could be expected considering the fact that numerous tests proved that bacteria are more sensitive to the antibiotic compared with fungi (HUGO *et al.*, 1983). The reason for different sensitivity between the fungi and bacteria can be found in different transparency of the cell wall (YANG *et al.*, 1999). The cell wall of the gram-positive bacteria consists of peptidoglucans (mureins) and teichoic acids, the cell wall of the gram-negative cells consists of lipo polysaccharides, and lipopoliproteins (HUGENHOLTZ, 2002; 1982; JEAN VAN HEIJNOORT, 2001) whereas the cell wall of fungi consists of polysaccharides such as hitchin and glucan (GRIFFIN, 1994).

Previous researches showed significant bioactive characteristics of similar lichens. GULLUCE *et al.* (2006) found out that the methanol extract of the lichen *Parmelia saxatilis* had a strong antimicrobial influence. Similar results were reported by CANDAN *et al.* (2007) for different extracts extracted from the lichen *P. sulcata*. RANKOVIĆ *et al.* (2007b) find an antimicrobial activity for the extracts of the lichens *P. caperata* and *P. pertusa*.

In this work, the antimicrobial activity of the lichens *Lecanora atra*, *L. muralis* and *Parmeliopsis ambigua* was researched for the first time. The obtained results indicated that the tested lichen extracts showed a significant antimicrobial influence in relation to the tested bacteria and fungi, which could be of significance for their use for pharmaceutical purposes.

CONCLUSIONS

Different lichen substances moderately and in some cases significantly inhibited the tested microorganisms, the majority of which are pathogens of man, animals, or plants. The obtained results showed that the tested lichen extracts had shown a significant antimicrobial effect in relation to the tested microorganisms. It can be useful in treatment of numerous diseases caused by these and similar microorganisms. Since there's a huge problem in the treatment of the infectious diseases, because the microorganisms had developed the resistance to numerous antibiotics, so the use of the active lichen extracts and lichen components could have an important role in their therapy.

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