Antimicrobial activity of *Rhodobryum ontariense*

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Abstract

The antimicrobial activity of dimethyl sulfoxide extract of moss *Rhodobryum ontariense* (Kindb.) Kindb. was evaluated by microdilution method against eight bacterial (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Enterobacter cloacae*, *Listeria monocytogenes, Bacillus cereus*, *Micrococcus flavus* and *Staphylococcus aureus*) and five fungal species (*Aspergillus versicolor*, *Aspergillus fumigatus*, *Penicillium funiculosum*, *Penicillium ochrochloron* and *Trichoderma viride*). The extract was proven to be active against all the bacteria and fungi tested but to varying degrees. It showed better inhibitory activity compared to the known antifungal drug against *T. viride* (*MIC* 100 and 200 μg/ml, respectively). This finding implies that *R. ontariense* could be considered as a promising material for natural antifungal products.

Keywords: Moss, *Rhodobryum ontariense*, DMSO extract, Antibacterial and antifungal activity.

Available online at the Journal website: http://www.ache.org.rs/HI/

RESEARCH NOTE

UDC 582.32:547.914:615.322.07

Hem. Ind. **66** (3) 381–384 (2012)

doi: 10.2298/HEMIND111107100P

Bryophytes are the second largest group of terrestrial plants with an estimated number of 20,000 to 28,000 species worldwide [1]. The small size and biomass of these plants have caused them to be neglected for wider use. In fact, they have been long considered to be economically insignificant except for mosses used in packing, plugging and decoration.

However, the antibiotic influence of liverworts *Anthoceros*, *Conocephalum*, *Jungermannia*, *Marchantia* and *Riccia*, and mosses *Atrichum*, *Dicranum*, *Mnium*, *Polytrichum* and *Sphagnum* has been known for a long time [2]. Many bryophytes have been investigated for their antimicrobial activity [3–8]. Extracts of mosses *Meteorium buchananii* and *Meteorium subpolytrichum* have been showed to be highly effective to *Staphylococcus aureus* [9]. Zinmeister and Asakawa have stated that bryophytes are one of the important sources of antibiotics and biologically active, naturally occurring compounds [10–11]. On the other hand, the antibiotic activity of bryophytes, as another plant groups, varies from species to species. It also depends on the age of the plant, season of collection and the ecological niche [12].

In Serbia the genus *Rhodobryum* is represented by two species (*Rhodobryum roseum* and *Rhodobryum ontariense*), which are not widely distributed [13–14] (Figure 1). The aim of this study was to find out if *R. ontariense* could be considered as an interesting source of antimicrobial agents.

Figure 1. The moss Rhodobryum ontariense in situ (the Deliblato Sands, Serbia).

EXPERIMENTAL

Plant material

The moss *Rhodobryum ontariense* (Kindb.) Kindb. (Bryaceae), collected near Belgrade (the Deliblato Sands, Serbia) in March 2007, was dried and stored at room temperature (25±2 °C) before extraction. A voucher specimen has been deposited in the Herbarium of the Institute of Botany, University of Belgrade, Serbia (bryophyte collection BEOU No. 4708).

Extraction procedure and microorganisms used

The green leaves of gametophyte tips used for extraction were dried by airflow at room temperature.

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Paper received: 7 November, 2011
Paper accepted: 28 November, 2011

The extraction was performed by slight rehydratation of dried plant material in dimethyl sulfoxide (DMSO) (10 mg of the material per 1 ml of the solvent), left in the dark for 12 h, and the extract was filtered with a cellulose-acetate membrane (0.45 μm). The DMSO extract was chosen because of its property to be inert and to not show any activity compared to other solvents such as methanol or ethanol, for example. Indeed, for the reason of verifying its toxicity, DMSO alone (in the amount used to dissolve samples) was also tested in this antimicrobial screening and found not to interfere with obtained results.

For adequate bioassays, eight bacteria, four Gram-negative bacteria: *Escherichia coli* ATCC 35210, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311 and *Enterobacter cloacae* (human isolate), and four Gram-positive bacteria, *Listeria monocytogenes* NCTC 7973, *Bacillus cereus* (human isolate), *Micrococcus flavus*ATCC 10240 and *Staphylococcus aureus* ATCC 6538, and five fungi, *Aspergillus versicolor* ATCC 11730*, Aspergillus fumigatus* ATCC 9142*, Penicillium funiculosum* ATCC 36839*, Penicillium ochrochloron* ATCC 9112 and *Trichoderma viride* IAM 5061, were used. The bacteria were maintained on Mueller–Hinton agar (MH) and Lysogeny broth medium (LB) and the micromycetes on malt agar (MA). Cultures were stored at 4 °C and subcultured once a month [15].

Screening of antimicrobial activity

The modified microdilution technique was used to screen antimicrobial activity [16,17]. Bacterial species were cultured overnight at 37 °C in LB medium. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The fungal and bacterial cell suspensions were adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μl per well. The inocula were stored at 4 °C for further use. Dilutions of the inocula were cultured on solid MH for bacteria and solid MA for fungi to verify the absence of contamination and to check the validity of the inocula.

Determination of minimal inhibitory concentrations (MICs) was performed by a serial dilution technique using 96-well microtitre plates. The extract was dissolved in DMSO (10 mg/ml) and added in broth medium with inoculum. The microplates were incubated for 48 h at 37 °C for bacteria and for 5 days at 28 °C for fungi. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs [17,18]. The minimal bactericidal (MBCs) and fungicidal (MFCs) concentrations were determined in triplicate by serial subcultivation of 2 μl into microtitre plates containing 100 μl of broth per well and further incubation for 48 h at 37 °C or 5 days at 28 °C, respectively. The lowest concentration with no visible growth was defined as MBC/MFC respectively, indicating 99.5% killing of the original inoculum. Streptomycin/ampicillin and bifonazole/ketoconazole were used as positive controls for the bacteria and fungi, respectively.

RESULTS AND DISCUSSION

The DMSO extract was tested both for antibacterial and antifungal activities. The results for antibacterial activity are presented in Table 1; MIC and MBC values ranged between 1.00 and 3.00 mg/ml. The most sensitive species was *E. cloacae,* followed by *B. cereus* and *M. flavus.* The most resistant bacterium was *S. aureus*. Standard drugs streptomycin and ampicillin, used as positive controls, were active against all the bacteria. The range of MICs for streptomycin was from 0.05 to 0.10 mg/ml while MBCs were from 0.05 to 0.30 mg/ml; ampicillin showed lower antibacterial activity with MICs values between 0.10 and 0.30 mg/ml and MBCs values between 0.15 and 0.50 mg/ml.

Table 1. In vitro antibacterial activity of R. ontariense (MIC and MBC in mg/ml)

Bacterium	DMSO extract	Streptomycin	Ampicillin
S. aureus	3.00/3.00	0.10/0.10	0.10/0.10
B. cereus	1.00/2.00	0.05/0.05	0.10/0.10
M. flavus	1.00/2.00	0.05/0.10	0.10/0.15
L. monocytogenes	2.00/3.00	0.15/0.30	0.15/0.30
P. aeruginosa	2.00/2.00	0.10/0.20	0.30/0.50
E. cloaceae	1.00/1.00	0.10/0.20	0.15/0.20
S. typhimurium	2.00/2.00	0.10/0.20	0.10/0.20
E. coli	2.00/2.00	0.10/0.20	0.15/0.20

The extract showed better antifungal activity than antibacterial (Table 2); MICs were ranged between 0.10 and 0.50 mg/ml and MFCs from 0.25 to 1.00 mg/ml. The most sensitive micromycete was *T. viride* in contrast to other tested fungi, which were similarly sensitive. Bifonazole possessed inhibitory activity of 0.10 to 0.20 mg/ml and fungicidal of 0.20 to 0.25 mg/ml, while ketoconazole showed lower antifungal activity with MICs of 0.20 to 2.50 mg/ml and MFCs of 0.5 to 3.00 mg/ml. The fact that extract showed better inhibitory activity against *T. viride* in comparison with bifonazole (MICs 0.10 and 0.20 mg/ml, and MFCs 0.25 and 0.25 mg/ml, respectively) is encouraging.

According to Borges-Argáez *et al*., MIC value of 100 to 200 μg/ml is a fair one for plant extract in the search of new anti-infectious agents [19]. Thereby, *T. viride*, known as a resistant species, has been recently recognized to be pathogenic in immunosuppressed human host [20]. Inferred from these results a potential use of the moss in bio-fungicides production can be assumed. It opens the problem of axenic farming of these threat species in Serbia for the purpose of bio-harvesting.

Table 2. In vitro antifungal activity of R. ontariense (MIC and MFC in mg/ml)

Fungae	DMSO extract	Bifonazole	Ketoconazole
T. viride	0.10/0.25	0.20/0.25	2.50/3.00
P. funiculosum	0.50/1.00	0.20/0.25	0.20/0.50
P. ochrochloron	0.50/1.00	0.15/0.20	0.20/0.50
A. fumigatus	0.50/1.00	0.15/0.20	0.20/0.50
A. versicolor	0.50/1.00	0.10/0.20	0.20/0.50

Acknowledgments

This work was supported by the Ministry of Science and Technological Development of the Republic of Serbia (Research grants Nos. 143015, 143031, 143049 and 173040).

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ИЗВОД

АНТИМИКРОБНА АКТИВНОСТ *Rhodobryum ontariense*

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У *in vitro* испитивању антимикробне активности диметил-сулфоксидног екстракта маховине *Rhodobryum ontariense* на осам бактеријских (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Enterobacter cloacae*, *Listeria monocytogenes*, *Bacillus cereus*, *Micrococcus flavus* и *Staphylococcus aureus*) и пет гљивичних сојева (*Aspergillus versicolor*, *Aspergillus fumigatus*, *Penicillium funiculosum*, *Penicillium ochrochloron* и *Trichoderma viride*) коришћена је модификована микродилуциона техника. Дати екстракт у различитој мери показао је активност на све тестиране организме. Његова активност на гљиву *T. viride* била је јача од активности коју је показао антифунгални лек бифоназол употребљен као позитивна контрола (МИК 100 и 200 μg/ml, редом). Овај рад указује на чињеницу да *R. ontariense* представља обећавајући извор природних производа са антифунгалном активношћу.

Кључне речи: Маховина • *Rhodobryum ontariense* • ДМСО екстракт • Антибактеријска и антифунгална активност