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ANTIBACTERIAL AND ANTIFUNGAL ACTIVITY OF THE LIVERWORT *(PTILIDIUM PULCHERRIMUM)* METHANOL EXTRACT

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Abstract - The antimicrobial activity of the methanol extract from the liverwort, *Ptilidium pulcherrimum* was evaluated against five bacterial and six fungal species. *In vitro* antibacterial activity was assessed by disc diffusion and microdilution methods. The extract showed a stronger effect against tested Gram (+) than Gram (-) bacteria. The antifungal activity of the methanol extract was tested using a microdilution method. The methanol extract showed strong antifungal activity. The best antifungal activity was achieved against *Trichoderma viride* compared to the synthetic fungicide bifonazol.

Keywords: Ptilidium pulcherrimum, methanol extract, antibacterial activity, antifungal activity, microdilution method, disc diffusion method

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INTRODUCTION

The liverwort *Ptilidium pulcherrimum* (Weber) Vainio is distributed in Fennoscandia and N. Russia, Iceland, the Caucasus, Siberia, Shensi and North America. It grows on the branches, trunks and exposed roots of deciduous shrubs or trees, rotting logs and rarely on rocks (Smith, 1999). This species is recorded in Serbia on the mountains Zlatar, Kopaonik, Crni Vrh and Tara.

Bryophytes have been used as medicinal plants in traditional medicine for a long time. They are used for treating various skin diseases and injuries, since they are believed to protect skin and open wounds from pathogenic microorganisms (Flowers, 1957).

The chemical constituents of liverworts have been investigated because this group contains cellular oil bodies which are composed of lipophilic terpenoids and aromatic compounds, in contrast to genuine mosses and hornworts. The phytochemistry of liverworts has been investigated from the aspects of chemotaxonomic and biological activity (Asakawa, 2001, 2004). A number of the isolated and identified compounds possess significant pharmacological activity.

Various kinds of biological activities of the compounds from bryophytes have been reported so far. Even in a single species, multiple types of activity have been reported. Thus, the liverworts *Marchantia tosana* and *Plagiochasma japonica* exhibited antifungal, antibacterial and antitumor activity, inhibition of superoxide release, inhibition of thrombin activity, and muscle relaxation (Lahlou et al., 2000). It has been shown that the ethanol extracts of *Plagiochila stephensoniana* were active against *Trichophyton mentagrophyes* and *Candida albicans,* and *Bacillus subtilis,* but not Gram (-) bacterium *E. coli* (Lorimers and Perry, 1993). A bioassay-guided separation of the antifungal constituents of *Marchantia polymorpha* led to the isolation of seven bis[bibenzyl]-type macrocycles (Niu et al., 2006).

No evidence of the use of *Ptilidium pulcherrimum* or related species in traditional medicine has been

found. Since literature data indicate that liverworts possess various kinds of pharmacological activities, the goal of our work was to evaluate the effect of an extract from this species against bacteria and microfungi.

MATERIAL AND METHODS

Plant material was collected from its native habitat (mountain Zlatar, Serbia) in September 2000. A voucher specimen of this species is kept in the Herbarium of the Institute of Botany and Botanical Garden "Jevremovac", Faculty of Biology, University of Belgrade (BEOU - 16116).

A sample (10 g) was dried by airflow at room temperature, powdered and extracted with 80% methanol (100 x 2 ml) for 24 h at 40˚C. The extract was filtered with a cellulose-acetate membrane (0.45 μm). The filtrate was evaporated until dry with a rotary evaporator and 80 mg of dry extract was dissolved with 1 ml dimethyl sulfoxide (DMSO) (Ilhan et al., 2006).

Tests for antibacterial and antifungal activity

The following bacterial species were used: *Staphylococcus aureus* (ATCC 25923), *Micrococcus flavus* (ATCC 9341), *Echerichia coli* (ATCC 25922), *Enterobacter cloacae* (human isolate), *Salmonella typhimurium (*ATCC 13311). The bacterial species were cultured overnight at 37°C in LB medium. Inoculum suspensions containing ~10^6 cells/ml were used for the experiments. The antibacterial assays were carried out by the modified disc-diffusion method (Verpoorte et al., 1983) and microdilution method (Hanel and Raether, 1988; Daouk et al., 1995).

The following fungi were used: *Aspergillus niger* (ATCC 6275), *A. ochraceus* (ATCC 12066), *A. versicolor* (ATCC 11730), *A. flavus* (ATCC 9170), *Penicillium funiculosum* (ATCC 10509), *Trichoderma viride* (IAM5061).

Disc diffusion method

A sterile filter disc (diameter 4 mm, Whatman paper No. 3) was placed in Petri dishes (diameter 90 mm) filled with Mueller-Hinton agar and seeded with 0.3 ml of the test organism. The disc was impregnated with test concentrations (0.05-2 mg/disc) of the compounds investigated dissolved in DMSO. The zones of growth inhibition around the discs were measured after 24h of incubation at 37°C. Each microorganism was tested in triplicate and the solvent (DMSO) was used as a control, while streptomycin was used as a positive control.

Table 1. Antibacterial activity of the *Ptilidium pulcherrimum* methanol extract assessed by the disc diffusion method.

Zone of inhibition (mm)		
Bacteria	*P. pulcherrimum* (2 mg/disc)	Streptomycin (0.02 mg/disc)
Staphylococcus aureus	12.3	40.0
Micrococcus flavus	13.3	55.0
Escherichia coli	-	26.3
Enterobacter cloacae	-	-
Salmonella typhimurium	-	35.3

Microdilution method

The modified microdilution technique was used to obtain quantitative data for the compounds investigated, (Hanel and Raether, 1988; Daouk et al., 1995). Bacterial species were cultured overnight at 37°C in LB medium. The inoculum suspension was adjusted with sterile saline to a concentration of approximately 1.0 x 10^5 in a final volume of 100 μl per well. The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (vol/vol) and adjusted with sterile saline to a concentration of 1.0 x 10^5 in a final volume of 100 μl/ml. The inocula were stored at +4° C for further use. Dilutions of the inocula were cultured on solid MH for bacteria and MA for fungi to verify the absence of contamination and to check the validity of the inoculum.

Table 2. Antibacterial activity of the *Ptilidium pulcherrimum* methanol extract assessed by the microdilution method.

	P. pulcherrimum (mg/ml)		Streptomycin (mg/ml)	
Bacteria	MIC	MBC	MIC	MBC
Staphylococcus aureus	10	20	0.05	0.1
Micrococcus flavus	10	20	0.05	0.1
Escherichia coli	20	>20	0.1	0.2
Enterobacter cloacae	10	20	0.1	0.3
Salmonella typhimurium	-	-	0.1	0.4

Minimum inhibitory concentration (MIC) determination was performed by a serial dilution technique using 96-well microtiter plates. The investigated compounds were dissolved in broth medium with inoculum to achieve the desired concentrations (0.05-20 mg/ml). Microplates were incubated for 48 h at 37° C for bacteria and 72 h at 28° C for fungi. The lowest concentrations without visible growth (a binocular microscope was used) were defined as concentrations which completely inhibited bacterial or fungal growth (MICs). The minimum bactericidal concentrations (MBCs) and minimum fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µl into microtiter plates containing 100 µl of broth per well and further incubation for 24/72 h at 28/37° C. The lowest concentration with no visible growth was defined as MBC/MFC, indicating = 99.5% killing of the original inoculum. DMSO was used as a control, while streptomycin and bifonazole were used as a positive control.

RESULTS AND DISCUSSION

In this work, the antibacterial and antifungal activity of the methanol extract of *Ptilidium pulcherrimum* was tested against Gram (+) and Gram (-) bacteria and 6 micromycetes. For comparison of the antimicrobial activity a synthetic antibiotic Streptomycin was used, while for the antifungal activity a synthetic fungicide Bifonazol was used. Using the disc diffusion method and a concentration of 2 mg per disc, the results differed to a degree. The extract showed activity against *Staphylococcus aureus* and *Micrococcus flavus,* while the other organisms were resistant (Tab. 1). It is also evident that the antibacterial activity of Streptomycin was higher than the tested extract.

Table 3. Antifungal activity of the *Ptilidium pulcherrimum* methanol extract assessed by the microdilution method.

	Concentration of extract and bifonazol (mg/ml)			
	P. pulcherrimum		Bifonazol	
Fungi	MIC	MFC	MIC	MFC
Trichoderma viride	0.5	2.5	1.0	1.0
Aspergillus flavus	0.5	2.5	0.1	0.1
Aspergillus versicolor	2.5	5.0	0.1	0.1
Aspergillus ochraceus	2.5	5.0	0.5	1.0
Aspergillus niger	2.5	5.0	0.1	0.1
Penicillium funiculosum	0.5	2.5	0.5	1.0

According to the obtained results it is evident that the extract of *Ptilidium pulcherrimum* at a concentration of 20 mg/ml has high antibacterial activity. Using the microdilution method MBC was observed against most of the investigated species, while inhibitory activity against *Escherichia coli* was observed. Only *Salmonella typhimurium* was resistant at the concentration tested (Tab. 2).

Antifungal activity of the methanol extract of *Ptilidium pulcherrimum* was tested using a microdilution method. The activity was tested against 6 micromycetes (Table 3). *Ptilidium pulcherrimum* had a fungicidal effect on one half

of tested organisms. Regarding *Trichoderma viride,* this concentration showed a better inhibitory effect than the synthetic fungicide Bifonazol. Similar results were obtained with *Penicillium funiculosum*. The concentration of 2.5 mg/ml showed a significant fungicidal effect against one half of the tested organisms, while against the other half of the species a higher (double) concentration was needed (Tab. 3).

From various investigations it can be seen that the extracts of liverworts show considerable antimicrobial effect against micromycetes and G (+) bacteria, while their activity against G (-) bacteria is weak. A similar effect is given in our work. Thus, the methanol extract of *Ptilidium pulcherrimum* showed a moderate antibacterial and strong antifungal activity. The results indicate that the methanol extract of this species (20 mg/ml) have bacteriostatic activity against *Escherichia coli,* while against *Staphylococcus aureus*, *Micrococcus flavus* and *Enterobacter cloacae* the extract showed bactericidal activity. There is no activity against *Salmonella typhimurium*. The micromycetes tested were more sensitive than the bacterial species (MIC 0.5-2.5 mg/ml and MFC 2.5-5.0 mg/ml). Moreover, *Trichoderma viride*, which is known as a resistant species, was also very sensitive to this extract. Bifonazol showed almost the same fungistatic activity, while its fungicidal activity was slightly higher than the methanol extract of the analyzed liverwort.

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АНТИБАКТЕРИЈСКА И АНТИФУНГАЛНА АКТИВНОСТ МЕТАНОЛНОГ ЕКСТРАКТА ЈЕТРЕЊАЧЕ *PTILIDIUM PULCHERRIMUM*

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Антибактеријско и антифунгално дејсто метанолног екстракта јетрењаче *Ptilidium pulcherrimum* испитивано је на 5 врста бактерија и 6 микромицета. За испитивање *in vitro* антимикробног дејства екстракта коришћена је метода микродилуције на микротитрационим плочама и метода дифузије са филтер дискова. Екстракт је показао јачи ефекат на тестиране Грам (+), него на Грам (-) бактерије. Антифунгална активност метанолног екстракта испитана је микродилуционом методом. Тестирани екстракт показао је јак антифунгални потенцијал на *Trichoderma viride,* Бифоназол је показао скоро идентичну фунгистатичку активност, док је његова фунгицидна активност била нешто виша у поређењу са метанолним екстрактом анализиране јетрењаче.