

ANTIMICROBIAL ACTIVITY OF METHANOL EXTRACTS OF *ABIETINELLA ABIETINA*, *NECKERA CRISPA*, *PLATYHYPNIDIUM RIPAROIDES*, *CRATONEURON FILICINUM* AND *CAMPYLIUM PROTENSUM* MOSSES

DANKA BUKVIČKI¹*, M. VELJIĆ¹, MARINA SOKOVIĆ², SLAVICA GRUJIĆ¹ and P. D. MARIN¹

¹ University of Belgrade, Faculty of Biology, Institute of Botany and Botanical Garden "Jevremovac", 11000 Belgrade, Serbia

² University of Belgrade, Institute for Biological Research "Siniša Stanković", 11000 Belgrade, Serbia

Abstract – The antibacterial and antifungal activity of methanol extracts of the genuine mosses *Abietinella abietina*, *Neckera crispa*, *Platyhypnidium riparoides*, *Cratoneuron filicinum* var. *filicinum* and *Campylium protensum* were evaluated. Antibacterial activity was tested against Gram (+) *Staphylococcus aureus*, *Micrococcus flavus*, *Bacillus cereus* and Gram (-) bacteria *Escherichia coli* and *Salmonella typhimurium*. Antifungal activity was tested using micromycetes *Trichoderma viride*, *Penicillium funiculosum*, *Penicillium ochrochloron*, *Aspergillus flavus*, *A. niger* and *A. fumigatus*. The methanol extracts of all moss species showed an antimicrobial effect against the tested microorganisms. Significant antibacterial effect was achieved for *Cratoneuron filicinum* and *Neckera crispa*. The most sensitive bacteria were *Bacillus subtilis* and *Micrococcus flavus*. *Abietinella abietina* and *Neckera crispa* showed an antifungal effect against micromycetes *Trichoderma viride*, *Penicillium ochrochloron*, *P. funiculosum* and *Aspergillus flavus*.

Key words: Mosses, methanol extracts, antibacterial activity, antifungal activity

INTRODUCTION

Bryophytes are useful plants as sources of natural products since they grow everywhere in the world (Asakawa, 2008). Mosses are a rich source of secondary metabolites with antimicrobial activity (Asakawa, 1981, 2007). The secondary metabolites identified from mosses belong to terpenoids, flavonoids and bibenzyls, but they are also rich in other compounds such as fatty acids (Borel et al., 1993), acetophenols (Lorimers and Perry, 1993), and arylbenzofurans (Von Reusz and König, 2004).

Antimicrobial activity is related to the specific chemical composition, structural configuration of compounds, functional groups, as well as potential synergistic or antagonistic interactions between

compounds. A large number of antimicrobial agents have been isolated in the last decade and used to treat diseases caused by microorganisms. To date, over several hundred new compounds have been isolated from bryophytes and their structures have been elucidated. The biological characteristics of the terpenoids and aromatic compounds isolated from liverworts show antibacterial and antifungal activity, cytotoxic activity, antiHIV, insect antifeedant activity, and superoxide anion radical release activity (Asakawa, 2008).

Terpenoids, phenolic and volatile constituents have also been investigated in some bryophytes. Many of the terpenoids were described and isolated mainly from liverworts (Saritas, 2001). Monoterpenes such as α -pinene, β -pinene, camphor, sabinene,

myrcene, α -terpinene and limonene give a characteristic smell to mosses, and some of them show antimicrobial activity.

The antibacterial activity of flavonoids has been reported (Xu and Lee, 1999). Among the flavonoids examined, four flavonols (myricetin, datiscetin, kaempferol and quercetin) and two flavones (flavone and luteolin) exhibited inhibitory activity against methicillin-resistant *Staphylococcus aureus* (MRSA). Myricetin was also found to inhibit the growth of the multidrug-resistant *Burkholderia cepacia*, vancomycin-resistant enterococci (VRE). Seven pure flavonoids were isolated and identified from five moss species (Basile et al., 1999). All the flavonoids showed good antimicrobial activity against the tested bacteria and the highest activity that of saponarine. Some of these flavonoids were shown to have pronounced antibacterial effects. Biflavonoids in mosses have also been reported as possible agents against microorganisms (Lopez-Saez, 1996).

Research into the antimicrobial activity of mosses has increased in the last decade (Basile et al., 2003; Dulger et al., 2005; Ilhan et al., 2006; Sabovljević et al., 2006, 2010, 2011; Veljić et al., 2008, 2009; Altuner, et al. 2009; Savaroglu et al., 2011).

The aim of this work was to test the antimicrobial activity of methanol extracts of the moss species *Abietinella abietina* (Hedw.) M. Fleisch, *Neckera crispa* Hedw., *Platyhypnidium riparoides* (Hedw.) Dixon, *Cratoneuron filicinum* (Hedw.) Spruce var. *filicinum* and *Campylium protensum* (Brid.) Kimbd. as a starting point for further investigations of the isolated individual components responsible for antimicrobial activity.

MATERIALS AND METHODS

The plants were dried at room temperature and pulverized into a fine powder using an electric blender. The powdered material (5 g) was extracted with 200 ml of methanol for 24 h at room temperature. After 24 h, the mixture was filtered through Whatman filter paper. The extracts were prepared in a rotary

evaporator (Laborota 4001, Heidolph). The obtained extracts were stored at +4°C until further tests.

Methanol extracts of the following species were used in this experiment: *Abietinella abietina* (Hedw.) M. Fleisch, collected 13.06.2006, locality: canyon Derventa near a lake (Voucher No. 16184); *Neckera crispa* Hedw., collected 13.06.2006, locality: Derventa, (Voucher No. 16180); *Platyhypnidium riparoides* (Hedw.) Dixon, collected 14.06.2006, locality: Rača Lađevac, (Voucher No. 16181); *Cratoneuron filicinum* (Hedw.) Spruce var. *filicinum*, collected 20.06.2007, locality: Rača Lađevac, (Voucher No. 16182) and *Campylium protensum* (Brid.) Kimbd., collected 13.06.2006, locality: Derventa, (Voucher No. 16183). All species were identified by M. Veljić.

The extracts were tested against the following bacteria: *Staphylococcus aureus* (ATCC 6538), *Micrococcus flavus* (ATCC 10240), *Bacillus cereus* (clinical isolate) and Gram (-) bacteria *Escherichia* (ATCC 35218), and *Salmonella typhimurium* (ATCC 6538) synthetic antibiotic streptomycin. Antifungal activity was tested using the following species: *Trichoderma viride* (ATCC IAM 5061), *Penicillium funiculosum* (ATCC 10509), *Penicillium ochrochloron* (ATCC 9112), *Aspergillus flavus* (ATCC 9170), *Aspergillus niger* (ATCC 6275), *Aspergillus fumigatus* (human isolate) and synthetic fungicides, canesten, ketoconazole, prochloraz and fundazol. The microfungi were maintained on malt agar and the cultures stored at 4°C and sub-cultured once a month (Booth, 1971). In order to investigate the antifungal activity of the extracts, a modified microdilution technique was used (Hanel and Raether, 1988; Espinel-Ingroff, 2001). The fungal spores were washed from the surface of agar plates with sterile 0.85% saline containing 0.1% Tween 80 (v/v). The spore suspension was adjusted with sterile saline to a concentration of approximately 1.0×10^5 in a final volume of 100 μ l per well. The inocula were stored at 4°C for further use. Dilutions of the inocula were cultured on solid malt agar to verify the absence of contamination and to check the validity of the inoculum.

Table 1. Antibacterial activity of methanol extracts of moss (mg/ml) and streptomycin.

Mosses Bacteria		<i>A. abietina</i>	<i>N. crispa</i>	<i>P. riparoides</i>	<i>C. filicinum</i>	<i>C. protensum</i>	streptomycin ¹
<i>S. aureus</i>	MIC*	5.00	2.50	2.50	5.00	5.00	0.05
	MBC**	5.00	5.00	5.00	5.00	5.00	0.05
<i>B. cereus</i>	MIC	2.50	2.50	2.50	2.50	2.50	0.02
	MBC	2.50	2.50	2.50	2.50	5.00	0.05
<i>M. flavus</i>	MIC	2.50	2.50	2.50	2.50	2.50	0.02
	MBC	2.50	5.00	2.50	2.50	5.00	0.05
<i>E. coli</i>	MIC	5.00	2.50	5.00	2.50	5.00	0.05
	MBC	5.00	5.00	5.00	2.50	5.00	0.05
<i>S. typhimurium</i>	MIC	5.00	5.00	5.00	5.00	5.00	0.05
	MBC	5.00	5.00	5.00	5.00	5.00	0.05

*Minimum inhibitory concentration (MIC), **Minimum bactericidal concentration (MBC), ¹commercial antibiotic

Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The samples investigated were added in broth Malt medium with inoculum. The microplates were incubated for 72 h at 28°C, respectively. The lowest concentrations without visible growth (with a binocular microscope) were defined as MICs.

The fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µl into microtiter plates containing 100 µl of broth per well and further incubated for 72 h at 28°C. The lowest concentration with no visible growth was defined as MFC causing 99.5% killing of the original inoculum. The commercial fungicide, Bifonazole, was used as a positive control (1 g active compound in 100 ml diluted ethanol).

Bacterial and micromycetes strains were collected from the Department of Plant Physiology, Institute for Biological Research "Siniša Stanković", Belgrade, Serbia.

As a solvent, dimethylsulfoxide (DMSO) was used (5%). Dry extract dissolved in 5% DMSO was used in further work in the study of antimicrobial activity.

RESULTS AND DISCUSSION

The results of antibacterial activity of methanol extracts are presented in Table 1. All extracts showed bactericidal activity at concentrations of 5 mg/ml. The strongest activity was shown by the extract of *N. crispa* against all tested bacteria (MIC 2.50 mg/ml), except against *Salmonella typhimurium* (MIC 5 mg/ml). The antibacterial effect of the methanol extracts was higher against G (+) bacteria *Bacillus cereus* and *Micrococcus flavus*. The extract of *Cratoneuron filicinum* showed an inhibitory effect at 2.5 mg/ml against *Escherichia coli*, *Bacillus cereus* and *Micrococcus flavus*.

To our best knowledge, no previous reports about the influence of *Abietinella abietina*, *Neckera crispa*, *Platyhypnidium riparoides* and *Campylium protensum* extracts on bacteria and micromycetes are

Table 2. Antifungal activity of methanol extracts of mosses and commercial fungicides (mg/ml).

Micromycetes		mosses					fungicides			
		1.	2.	3.	4.	5.	canesten	ketoconazole	prochloraz	fundazol
<i>T. viride</i>	MIC*	1.25	1.25	10.00	5.00	5.00	0.005	0.010	0.010	<0.005
	MFC**	2.50	2.50	20.00	10.00	10.00	0.010	0.025	0.025	<0.005
<i>P. ochrachloron</i>	MIC	1.25	1.25	2.50	2.50	2.50	0.005	0.010	0.005	<0.005
	MFC	2.50	2.50	5.00	2.50	2.50	0.010	0.025	0.010	<0.005
<i>P. funiculosum</i>	MIC	1.25	1.25	5.00	2.50	2.50	0.100	0.005	0.005	<0.005
	MFC	2.50	2.50	5.00	5.00	5.00	>0.100	0.010	<0.005	<0.005
<i>A. flavus</i>	MIC	1.25	1.25	10.00	5.00	2.50	0.050	<0.005	0.010	<0.005
	MFC	2.50	2.50	20.00	10.00	2.50	0.050	<0.005	0.010	<0.005
<i>A. fumigatus</i>	MIC	5.00	5.00	10.00	5.00	5.00	0.100	0.010	0.010	<0.005
	MFC	10.00	10.00	20.00	10.00	5.00	>0.100	0.025	0.010	<0.005
<i>A. niger</i>	MIC	5.00	5.00	10.00	5.00	5.00	0.100	0.025	0.005	<0.005
	MFC	5.00	5.00	10.00	10.00	10.00	>0.100	0.025	<0.005	<0.005

1. *A. abietina*, 2. *N. crispa*, 3. *P. riparoides*, 4. *C. filicinum*, 5. *C. protensum*

*Minimum inhibitory concentration (MIC); **Minimum fungicidal concentration (MFC).

available. Singt et al. (2006) reported that the ethanol extract of *Cratoneuron filicinum* showed activity against six of eleven investigated bacteria (MIC 1.56-3.12 µg/ml) and antifungal activity against yeast *Candida albicans* (MIC 3.12 µg/ml).

Earlier studies have demonstrated that the *Cratoneuron filicinum* show a considerable effect in heart disease (Asakawa, 2007). Recent studies have also demonstrated the activity of *Palustriella commutata* (Hedw.) (Syn. *Cratoneuron commutatum*). The methanol extract of this species showed activity against five bacteria (*Micrococcus luteus*, *Yersinia enterocolitica*, *Bacillus cereus*, *Klebsiella pneumoniae* and *Escherichia coli*) (Ilhan et al. 2006).

The moss extracts showed bacteriostatic and bactericidal activity. The bacteria with the highest

sensitivity were *Bacillus subtilis* and *Micrococcus flavus*. The lowest concentration of extract showed bactericidal effect at 2.5 mg/ml (MIC). Synthetic antibiotic streptomycin showed an inhibitory effect at 0.02 mg/ml against the bacteria *Bacillus cereus* and *Micrococcus flavus*. The most sensitive bacteria were *Bacillus cereus* and *Micrococcus flavus*. The strongest antibacterial activity was shown by the *Neckera crispa* extract.

The results of the antifungal activity of the tested extracts are given in Table 2. The analyzed extracts expressed moderate fungistatic and fungicidal effects. The strongest antifungal potential was shown by the extracts of *Abietinella abietina* and *Neckera crispa*. For the micromycetes *Trichoderma viride*, *Penicillium ochrochloron*, *P. funiculosum* and *Aspergillus flavus* the MIC value was 1.25 mg/ml, while

the MFC was 2.5 mg/ml. The lowest antifungal activity was observed for the extract of *Platyhypnidium riparoides*. MIC ranged between 2.5-10 mg/ml, while MFC ranged between 5.00 to 20.00 mg/ml. The most resistant micromycete was *Aspergillus fumigatus* (MIC 5.00-10.00 mg/ml; MFC 5.00-20.00 mg/ml), while the most sensitive was *Penicillium ochrochloron* (MIC 1.25-2.50 mg/ml; MFC 2.50-5.00 mg/ml).

The extracts showed lower activity in comparison with the commercial fungicides canesten, ketoconazole, prochloraz and fundazol.

Based on the results of antibacterial and antifungal activity of moss extracts it is evident that the extracts show a biological activity. The antifungal activity of the analyzed moss species was higher than their antibacterial activity.

Mosses could be sources of new antibacterial, and especially antifungal agents. The isolation of biologically active substances from moss extracts could be useful in further investigations of agents providing protection against pathogenic microorganisms.

Acknowledgments - This research was supported by a grant from the Ministry of Education and Science of Serbia (Project No. 173029 and 173032).

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