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Full Length Research Paper

Comparison of extract bio-activities of *in-situ* and *in vitro* grown selected bryophyte species

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The antimicrobial activity of DMSO extracts of three bryophyte species, two mosses and a liverwort (*Atrichum undulatum* (Hedw.) P. Beauv., *Marchantia polymorpha* L. ssp. *ruderalis* Bischl. and Boisselier, *Physcomitrella patens* (Hedw.) Bruch and Schimp.) grown in nature and in axenic culture was evaluated by microdilution method against eight bacterial species (*Escherichia coli* ATCC 35210, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* ATCC 13311, *Enterobacter cloacae* (human isolate), *Listeria monocytogenes* NCTC 7973, *Bacillus cereus* (human isolate), *Micrococcus flavus* ATCC 10240 and *Staphilococcus aureus* ATCC 6538). All investigated bryophyte extracts are proved to be active against all bacteria tested. In general, extracts made from material grown in nature express better anti-bacterial activity comparing to those made from material grown in laboratory conditions. Some of the bacteria react the same to both extracts and some even better to the extracts made from axenically grown material.

Key words: Bryophytes, antibacterial activity, *Atrichum undulatum*, *Marchantia polymorpha*, *Physcomitrella patens*.

INTRODUCTION

Bryophytes (liverworts, hornworts and mosses) expressed interesting bioactivities (Dulger at al., 2005; Chobot et al., 2006; Sabovljevic et al., 2006; Singh et al., 2007; Tonguc and Mercili 2007; Veljic et al., 2009). They are not common in the diet of other organisms and even more, most of the consumers avoid them. Besides antifiding effect, bryophytes are known to posses various relationships with microorganisms (protozoas, fungi, bacteria, algae) (Ando and Matsuo, 1984; Castaldo-Cobianchi et al., 1988; Asakawa, 1990; Basile et al., 1998; Sabovljevic et al., 2001) and contains a set of various known and unknown secondary metabolites (Xie and Lou, 2009).

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Bryophytes, as a diverse group, are chemically still incompletely unknown although many new compounds for science were described from them mainly from liverworts. They have use in ethno-medicine, rather rare comparing to vascular plants and rather few uses is known in some traditional medicine. The reports on biological activities of bryophyte extracts are of neglected and unknown potentials of these second biggest group of land plants with ca. 25,000 species and much more infra-taxa worldwide (Sabovljevic and Sabovljevic, 2008).

Uses of studied bryophytes in traditional medicine

The plants from the family Marchantiaceae are well-known traditional Chinese medicinal herbs extensively used to treat skin tumefaction, to protect the liver and to treat hepatitis, being also used as antipyretics (Chobot et

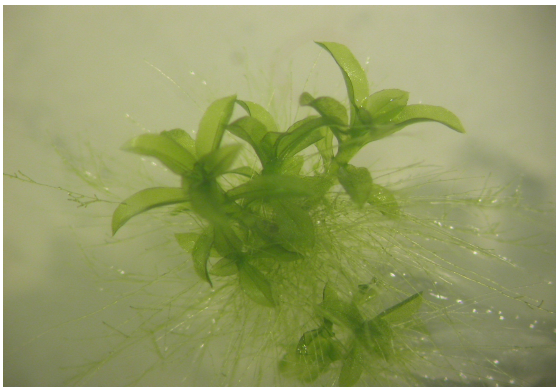

Figure 1. Axenical culture of *A. undulatum.*

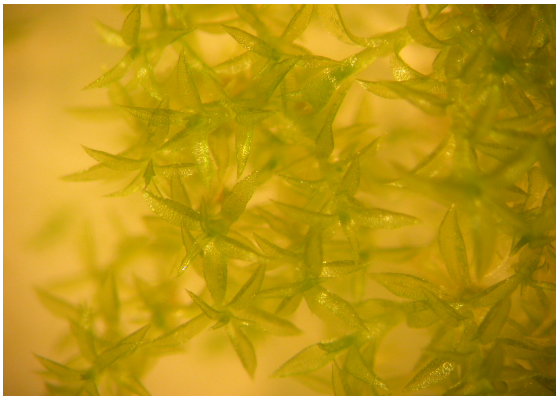

Figure 2. Axenical culture of *P. patens.*

al., 2006; Harris, 2008). Large numbers of Marchantiaceae plants occur in Chinese Guangxi Zhuang autonomous district such as *Marchantia polymorpha*, *Marchantia convoluta* and *Marchantia paleacea* and are used by local people. These species grow together and are difficult to distinguish one from another because of their similarity. Besides, many studies on the chemical constituents and bioactivities of *M. polymorpha s. lato* have been reported (Markham and Porter, 1974; Matsuo et al., 1985; Asakawa et al., 1987; Asakawa et al., 1990; Adam and Becker 1994; Rieck et al., 1997; Asakawa, 2001; Neelam and Padma 2008). The liverwort *M. polymorpha* has been used as a diuretic in traditional medicine for hundreds of years. Its pharmaceutical potency, however, was only recognized during the last three decades. Phytochemical investigations showed that besides terpenoids and flavonoids, bibenzyls and cyclic bis (bibenzyls) are also present in this plant. The most prominent examples of the group of bibenzyls are lunularic acid (Pryce, 1971), its decarboxylation product lunularine (Pryce, 1972) and the “prearomatic” precursor prelunularic acid (Ochta et al., 1983).

Atrichum labels are reported to be seen on Chinese medicines primarily as anti-bacterial and anti- inflammatory agents (Glime, 2007). McCleary and Walkington (1966) considered that non-ionized organic acids and polyphenolic compounds might contribute to the antibiotic properties of bryophytes and found among eighteen mosses also, *Atrichum* inhibited one or both of gram-positive and gram-negative bacteria. *Atrichum undulatum* was effective on everything tested except *Aerobacter aerogenes* and *Escherichia coli* confirmed also in our study. *Physcomitrella patens* is not known to be chemically tested or to posses traditional bioactivities.

Previously isolated classes of constituents in studied mosses

The liverwort *M. polymorpha* is known to posses many activities. However, since, it was recently under this name treated complex of species, it is not clear whether there is chemical distinction as well. However, it is well known by marclocyclic bis (bibenzyls)–various types of marchantin, some of which, besides antimicrobial are known to have anti-cancer effect (Asakawa et al., 1987; Chong et al. 2006; Asakawa et al., 2008)

The antibiotically active substances of *Atrichum* are considered to be polyphenolic compounds (McCleary and Walkington, 1966; Basile et al., 1999). It is also rich in flavonoids (Zinsmeister and Mues, 1980; Basile et al., 1999). Glycosides of three- and tetraoxygenated coumarins and polhydroxilated daphin coumarin were reported from *A. undulatum* (Jung et al., 1994; Chobot et al., 2008). Chobot et al. (2008) also reported relatively strong antioxidant activity.

Although, *P. patens* is bryophyte model system completely sequenced, the chemical constituents of this moss are not known.

The aim of this study was to compare bio-activities against bacteria of three randomly selected bryophyte species (one liverwort and two mosses) and to estimate if there is a difference among material grown in the wildness and axenically grown plants in laboratory controlled conditions.

MATERIALS AND METHODS

Plant material and extract preparation

The moss *A. undulatum* (Polytrichaceae) is widespread temperate species usually growing in forest flour. The tallous liverwort *M. polymorpha* ssp. *ruderalis* (Marchantiaceae) appears at the edges of the rivers and rivulets but also in wet and shaded urban and suburban sites. The ephemeral moss *P. patens* (Funariaceae) with short and quick life span appears in the spring time on the wet soils.

Plant material (shoots, apical thalli parts) was collected either from native habitats (Belgrade and surrounding) in spring 2009 or it was obtained from *in vitro* culture, so that were axenically farmed and disposed of co-habiting organisms.

Axenically cultures were established from spores (*A. undulatum* and *P. patens*) and from gemmae (*M. polymorpha* ssp. *ruderalis*) as described in Sabovljevic et al. (2009) (Figures 1 - 3). *A. undulatum*

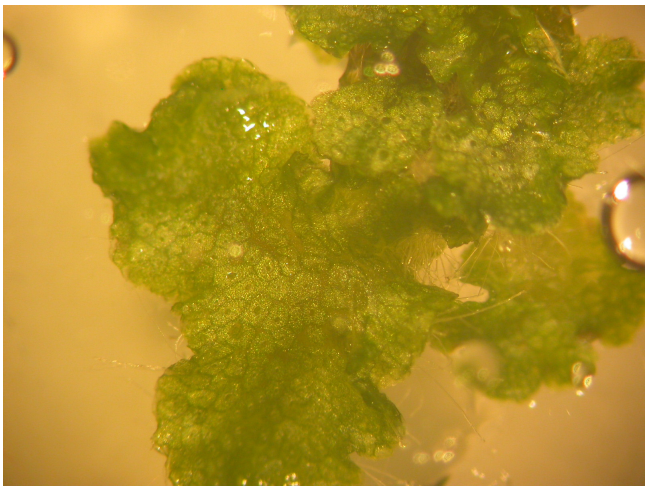

Figure 3. Axenical culture of *M. polymorpha* ssp. *ruderalis.*

and *M. polymorpha* gametophytes were grown on MS medium (Murashige and Skoog, 1962). Enriched with 0.1 M sucrose, while *P. patens* gametophytes were grown on BCD medium enriched with 0.1 M sucrose (Sabovljevic et al., 2009). In order to investigate how the environmental conditions influence bryophyte relationships with other species, they were grown either on solid or liquid MS/BCD medium, as indicated in the Table 1. The pH of the growth media was adjusted to 5.8 before autoclaving at 114°C for 25 min. Cultures were grown at 25 ± 2°C under the long day conditions (16/8 h of light to darkness) or under dark condition (24 h of dark), as indicated in the Table 1. Light was supplied by cool-white fluorescent tubes at a photon fluency rate of 47 µmol/m2s. Cultures were subcultured for a period of 4 - 6 weeks.

Voucher specimens of *A. undulatum*, *M. polymorpha* ssp. *ruderalis* and *P. patens* have been deposited in the BEOU bryophyte collection (No. 4463, 4556, 4509). The dimethyl sulfoxyde (DMSO) extracts of the studied bryophyte species were made from the specimens collected in the nature and their counter-parts axenically grown in *in vitro* conditions. The DMSO extract were made in both cases only from the green shoots (mosses) and apical thallus parts (liverwort). The DMSO extract from shoots of *A. undulatum*, *M. polymorpha* ssp. *ruderalis* and *P. patens* grown in nature, on 0.1 M sucrose enriched MS or BCD medium, under the light or dark conditions (average yields: 8.9, 7.6 and 7.2%).

All bryophyte samples (10 g) were dried by airflow at room temperature. They were then finely ground with a hammer mill and extracted separately with 1 ml of dimethyl sulfoxide (DMSO) for 24 h at room temperature. Extracts were filtered with cellulose-acetate membrane (0.45 µm).

Test for antibacterial activity

For the bioassays, eight bacteria were used: Gram-negative bacteria: *E. coli* (ATCC 35210), *Pseudomons aeruginosa* (ATCC 27853), *Salmonella typhimurium* (ATCC 13311), *Enterobacter cloacae* (human isolate); and Gram-positive bacteria: *Listeria monocytogenes* (NCTC 7973), *Bacillus cereus* (human isolate), *Micrococcus flavus* (ATCC 10240) and *Staphylococcus aureus* (ATCC 6538).

Bacterial species were cultured overnight at 37°C in LB medium. Inoculum suspensions containing ~10^6 cells/ml were used for experiments. The antibacterial assays were carried out by the modified microdilution method (Hanel and Raether, 1988; Daouk et al., 1995).

Microdilution method

In order to investigate the antimicrobial activity of the extracts tested, the modified microdilution technique was used (Hanel and Raether, 1988; Daouk et al., 1995). Bacterial species were cultured overnight at 37°C in LB medium. The bacterial cell 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 inocula were stored at +4°C for further use. Dilutions of the inocula were cultured on solid MH medium to verify the absence of contamination and to check the validity of the inoculums.

Minimum inhibitory concentrations (MICs) determination was performed by a serial dilution technique using 96-well microtiter plates. The compounds investigated were dissolved in DMSO (10 mg/mL) and added in broth medium with inoculum. The microplates were incubated for 48 h at 37°C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations which completely inhibited bacterial growth (MICs). The minimum bactericidal concentrations (MBCs) were determined by serial subcultivation of a 2 µL into microtitre plates containing 100 µL of broth per well and further incubation for 48 h at 37°C. The lowest concentration with no visible growth was defined as MBC indicating 99.5% killing of the original inoculum. DMSO was used as a control, while streptomycin and ampicilin were used as positive control (0.1 - 2 mg/ml).

RESULTS AND DISCUSSION

The antibacterial activities are reported in Table 1. All the extracts tested showed antibacterial activity but on different level. MIC ranged between 0.5 - 3.0 mg/ml while MBC is 1.0 - 3.0 mg/ml.

The best antibacterial activity is obtained for extract *A. undulatum* grown *in vitro*, on MS solid medium, under the long day conditions. The lowest antibacterial activity among all extracts tested was obtained for extract *M. polymorpha* ssp. *ruderalis* grown in nature with MIC 2.0 mg/ml and MBC 2.0 mg/ml. The most sensitive species was *B. cereus* and the most resistant bacteria was *L. moncytogenes*.

Standard drugs used as a positive control, streptomycin and ampicillin, were also active against all the bacteria. Range of MIC for streptomycin is 0.05 - 0.1 mg/ml and MBC 0.05 - 0.3 mg/ml, while ampicillin showed slightly lower antibacterial potential with MIC 0.1 - 0.3 mg/ml and MBC 0.15 - 0.5 mg/ml.

S. aureus equally reacted to extracts made from the nature and laboratory controlled plants grown in liquid medium of *M. polymorpha* ssp. *ruderalis* and solid medium in light condition of *A. undulatum*. The other nutrient combinations and light conditions as well as *P. patens* gave slightly lower inhibition. Effects of extracts from axenically grown material to *B. aureus* were better then those of natural plants for both *M. polymorpha* ssp. *ruderalis* and *A. undulatum.* No differences were recorded in *P. patens* extracts. *M. flavus*, *E. cloacae* better reacted to axenically grown material extracts of all three bryophyte species. *L. monocytogenes* has slightly better inhibition in extracts made from *A. undulatum* grown axenically in dark while *S typhimurium* has the highest values of inhibition with extracts of axenically

Table 1. Minimal inhibitory (MIC mg/ml) and bactericidal concentration (MBC mg/ml) of compounds tested.

Bacteria	*M. polymorpha*				*A. undulatum*			*P. patens*		streptomycin	ampicillin
	MS solid medium, light	MS liquid medium, light	MS solid medium, dark	nature	MS solid medium, light	MS solid medium, dark	nature	BCD solid medium, light	nature		
S. aureus	3.0	2.0	3.0	2.0	2.0	2.0	2.0	3.0	2.0	0.1	0.1
	3.0	3.0	3.0	2.0	2.0	3.0	2.0	3.0	2.0	0.1	0.15
B. cereus	0.5	1.0	1.0	2.0	0.5	0.5	1.0	1.0	1.0	0.05	0.1
	1.0	2.0	1.0	2.0	1.0	1.0	2.0	1.0	1.0	0.05	0.15
M. flavus	1.0	1.0	1.0	2.0	1.0	1.0	2.0	1.0	2.0	0.05	0.1
	2.0	1.0	2.0	2.0	1.0	2.0	2.0	2.0	2.0	0.1	0.15
L. monocytogenes	2.0	2.0	2.0	2.0	2.0	1.0	2.0	2.0	2.0	0.15	0.15
	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	3.0	0.3	0.3
P. aeruginosa	2.0	2.0	1.0	2.0	1.0	2.0	1.0	2.0	2.0	0.1	0.3
	3.0	2.0	2.0	2.0	1.0	2.0	2.0	2.0	2.0	0.2	0.5
E. cloacae	1.0	1.0	1.0	2.0	1.0	1.0	2.0	1.0	2.0	0.1	0.15
	1.0	1.0	2.0	2.0	1.0	1.0	2.0	1.0	2.0	0.2	0.2
Salmonella typhimurium	1.0	2.0	2.0	2.0	1.0	2.0	1.0	2.0	2.0	0.1	0.1
	2.0	3.0	2.0	2.0	1.0	2.0	2.0	2.0	2.0	0.2	0.2
E. coli	1.0	2.0	2.0	2.0	1.0	1.0	1.0	2.0	1.0	0.1	0.15
	2.0	2.0	2.0	2.0	1.0	1.0	2.0	2.0	2.0	0.2	0.2

grown *A. undulatum* in light condition. *E. coli* does not show significant differences on bryophytes extracts tested.

It can be seen that the growth of tested bacteria responded differently to the compounds tested, which indicates that different components may have different modes of action or that the metabolism of some bacteria is able to better overcome the effect of the agents or adapt to it.

Axenically growing bryophytes express to some extent the potential in using biotechnological processes. Since there is shown a variety of relationships of tested bryophyte extracts with selected bacteria, the target species and the growth condition should be adjusted to achieve the maximum (par wise selection).

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