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ANTIMICROBIAL ACTIVITY OF THE FRESHWATER BRYOZOAN *HYALINELLA PUNCTATA* (HANCOCK, 1850)

BORIS PEJIN[a*], JASMINA GLAMOCLIJA[b], ANA CIRIC[b], KSENIJA RADOTIC[a], VLATKA VAJS[c], VELE TESEVIC[d], ALEKSANDAR HEGEDIS[a], IVO KARAMAN[e], MLADEN HORVATOVIC[e], MARINA SOKOVIC[b]

[a]*Institute for Multidisciplinary Research, University of Belgrade, Kneza Viseslava 1, 11000 Belgrade, Serbia*

[b]*Mycological laboratory, Department of Plant Physiology, Institute for Biological Research "Sinisa Stankovic", University of* Belgrade, *Bulevar Despota Stefana 142, 11000 Belgrade, Serbia*

[c]*Center of Chemistry, Institute of Chemistry, Technology and Metallurgy, University of Belgrade, Njegoseva 12, 11000 Belgrade, Serbia*

[d]*Department of Organic Chemistry, Faculty of Chemistry, University of Belgrade, Studentski trg 16, 11000 Belgrade, Serbia*

[e]*Department of Biology and Ecology, Faculty of Sciences, University of Novi Sad, Trg Dositeja Obradovica 2, 21000 Novi Sad, Serbia*

The antimicrobial activity of the freshwater bryozoan *Hyalinella punctata* (Hancock, 1850) was tested by microdilution method against eight bacteria and eight fungi for the first time. All five crude extracts (hexane, acetone, dimethyl sulfoxide, methanol and water) showed good antibacterial and antifungal potential *in vitro* wherein the acetone extract was the most active (MICs 0.50-7.00 μg/ml and MBCs 2.50-10.00 μg/ml).

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1. Introduction

Freshwater bryozoans are sessile invertebrates that grow as colonies of connected zooids on submerged substrates. They live in lotic and lentic water and feed on suspended organic particles, which they capture with a whorl of ciliated tentacles called a lophophore. Bryozoans are classified into three major classes: primarily marine Gymnolaemata, marine Stenolaemata and freshwater Phylactolaemata. Unlike their marine relatives, phylactolaemates produce a kind of chitinous bud called statoblast which, under proper conditions, gives rise to new colonies [1]. Plumatellidae, the largest phylactolaemate family, contains about 55 species worldwide. Till date, ten species in two genera have been reported from Europe. The most speciose plumatellid genus is *Plumatella*, with about 40 species. *Hyalinella*, a genus closely related to *Plumatella*, currently contains only three species [2]. Their colonies are thick and transparent with less profuse branching than in *Plumatella* and produce only floatoblasts, while individual zooids are indistinct, usually arranged linearly and lack interzooidal septa. Although *Hyalinella punctata* (Hancock, 1850) is not a very frequent bryozoan species, it has been noticed in more European countries including Serbia [3,4].

With a few exceptions, bioactive natural products identified from marine bryozoan species so far are either alkaloids, sterols, bryostatins or heteroatom-containing compounds [5].

*Corresponding authors: borispejin@imsi.rs, brspjn@gmail.com

Antimicrobial activity have been found in bryozoans from Tasmania [6], the Mediterranean [7], the United Kingdom [8], south India [9], Japan [10], Scandinavia [11], the west coast of Canada [12] and from northern Puget Sound [13]. However, to date freshwater bryozoans have not been studied for antimicrobial activity. Antibiotic compounds may allow bryozoans to manipulate the composition of the bacterial film in their immediate vicinity. This could provide the bryozoans with some control over the types of organisms that are able to settle around them or on them [6-8] or may make the substrate more suitable for the settlement of bryozoan larvae [14,15].

On the other hand, antimicrobial agents of natural origin have provided the means to treat infections caused by microorganisms saving millions of individuals [16]. Nevertheless, during the last 20 years the problem of microbial resistance has emerged. Indeed, bacterial and fungal pathogens have evolved numerous defense mechanisms against the agents emphasizing the need for discovering more potent natural products as accessories or alternatives to existing therapies. In the course of our ongoing experiments toward the screening of antimicrobial activity of evolutionary simpler organisms [17-19], the bryozoan *H. punctata* was investigated. Herein, we report *in vitro* activity of its five extracts (hexane, acetone, dimethyl sulfoxide, methanol and water) against eight bacterial and eight fungal species.

2. Experimental

2.1. Animal material

The sample of *Hyalinella punctata* (Hancock, 1850) was collected in Belgrade (the river Danube, Serbia, 11.11.2011). Voucher specimen has been deposited in the Zoology Collection of the Department of Biology and Ecology of the University of Novi Sad, Serbia (BRY 003).

2.2. Extraction

After carefully cleaning from contaminants, the bryozoan sample was lyophilised. The dried parts of *H. punctata* were ground (2 g) and extracted thrice with hexane, acetone, dimethyl sulfoxide, methanol and hot water for 1 h at room temperature, respectively. The extracts were evaporated to dryness and stored at - 20 °C until further use.

2.3. Determination of antibacterial activity

The following Gram (-) /*Enterobacter cloacae* human isolate, *Escherichia coli* ATCC 35210, *Pseudomonas aeruginosa* ATCC 27853 and *Salmonella typhimurium* ATCC 13311/ and Gram (+) bacteria /*Bacillus cereus* clinical isolate, *Listeria monocytogenes* NCTC 7973, *Micrococcus flavus* ATCC 10240 and *Staphylococcus aureus* ATCC 6538/ were used. The organisms were obtained from Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Serbia. The antibacterial assay was carried out by microdilution method [20,21]. The bacterial suspensions were adjusted with sterile saline to a concentration of 1.0 x 10^5 CFU/ml. The inocula were prepared daily and stored at + 4 °C until use. Dilutions of the inocula were cultured on solid medium to verify the absence of contamination and to check the validity of the inoculum. All experiments were performed in duplicate and repeated thrice.

The minimum inhibitory and bactericidal concentrations (MICs and MBCs) were determined using 96-well microtitre plates. The bacterial suspension was adjusted with sterile saline to a concentration of 1.0 x 10^5 CFU/ml. The extracts for testing were added (1 mg/ml) in broth LB medium (100 µl) with bacterial inoculum (1.0 x 10^4 CFU per well) to achieve the wanted concentrations. The microplates were incubated at rotary shaker (160 rpm) for 24 h at 37° C. The lowest concentrations without visible growth (at the binocular microscope) were defined as concentrations that completely inhibited bacterial growth (MICs). The MBCs were determined by serial sub-cultivation of 2 µl into microtitre plates containing 100 µl of broth per well and further incubation for 24 h. The lowest concentration with no visible growth was defined as the MBC, indicating 99.5% killing of the original inoculum. The optical density of each well was measured at a wavelength of 655 nm by microplate manager 4.0 (Bio-Rad Laboratories) and compared with

a blank and the positive control. The antibitics streptomycin and ampicillin were used as positive controls (1 mg/ml in sterile physiological saline), while 5% solution of dimethyl sulfoxide was used as a negative control. All experiments were performed in duplicate and repeated thrice.

2.4. Determination of antifungal activity

The used fungi /*Aspergillus fumigatus* ATCC 1022, *Aspergillus niger* ATCC 6275, *Aspergillus ochraceus* ATCC 12066, *Aspergillus versicolor* ATCC 11730, *Candida albicans* human isolate, *Penicillium funiculosum* ATCC 36839, *Penicillium ochrochloron* ATCC 9112 and *Trichoderma viride* IAM 5061/ were obtained from Mycological Laboratory, Department of Plant Physiology, Institute for Biological Research "Sinisa Stankovic", University of Belgrade, Serbia. The micromycetes were maintained on malt agar and the cultures were stored at + 4° C and sub-cultured once a month [22]. The antifungal assay was carried out by modified microdilution technique. 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 µl 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. Minimum inhibitory concentration (MIC) determinations were performed by a serial dilution technique using 96-well microtiter plates. The examined extracts were added in concentration of 1 mg/ml in broth malt medium with inoculum. The microplates were incubated at rotary shaker (160 rpm) for 72 h at 28° C. The lowest concentrations without visible growth (at the binocular microscope) were defined as MICs. The fungicidal concentrations (MFCs) were determined by serial subcultivation of 2 µl of tested extracts dissolved in medium and inoculated for 72 h, into microtiter plates containing 100 µl of broth per well and further incubation 72 h at 28° C. The lowest concentration with no visible growth was defined as MFC indicating 99.5% killing of the original inoculum. The fungicides bifonazole and ketoconazole were used as positive controls (1–3500 µg/ml), while 5% solution of dimethyl sulfoxide was used as a negative control. All experiments were performed in duplicate and repeated thrice.

3. Results

The obtained data for antibacterial activity screening are presented in Table 1. The acetone extract exhibited maximum activity against five tested bacteria (MICs 0.50-7.00 µg/ml and MBCs 2.50-10.00 µg/ml) being the most active on Gram (+) bacteria *S. aurues* and *B. cereus*. On the other hand, the hexane extract showed maximum activity against three Gram (-) bacteria, namely *E. coli*, *P. aeruginosa* and *S. typhimurium* (MICs 1.00-1.50 µg/ml and MBCs 2.50 µg/ml). MICs and MBCs of the methanol extract were in broader ranges (10-60 µg/ml and 20-70 µg/ml, respectively); the most susceptible bacterium was Gram (+) *S. aureus* (MIC/MBC 10/20 µg/ml). Water and dimethyl sulfoxide extracts exhibited similar activity, but lower than the other tested (MICs 10-70 µg/ml and 20-70 µg/ml, respectively; MBCs 20-80 µg/ml and 40-80 µg/ml, respectively). Streptomycin possessed antibacterial activity at 12.50-150.00 µg/ml (MICs) and 25-300 µg/ml (MBCs); ampicillin exhibited MICs 100-300 µg/ml and MBCs 150-500 µg/ml. All examined extracts showed better antibacterial activity than ampicillin, and majority of them (acetone, hexane and methanol) were more active than streptomycin. Their antibacterial potential can be presented as follows: acetone > hexane > methanol > water > dimethyl sulfoxide.

Table 1. In vitro antibacterial activity of H. punctata.

Bacteria	Acetone extract*	Hexane extract*	Methanol extract*	Water extract*	Dimethyl sulfoxide extract*	Streptomycin*	Ampicilin*
S. aureus	0.50 2.50	10 20	10 20	20 40	40 50	50 100	100 150
B. cereus	0.50 2.50	10 20	20 50	10 20	40 50	12.50 25.00	100 150
M. flavus	7 10	10 30	20 40	70 80	70 80	25 50	100 150
L. monocytogenes	2.50 10.00	20 40	60 70	60 80	60 80	150 300	150 300
P. aeruginosa	5 10	1.00 2.50	20 40	10 20	20 40	50 100	300 500
S. typhimurium	5 10	1.00 2.50	20 40	20 40	20 50	50 100	100 200
E. coli	2.50 10.00	1.50 2.50	20 40	50 80	50 80	50 100	150 200
E. cloacae	2.50 10.00	20 40	20 40	60 80	60 80	50 100	150 200

* MIC/MBC μg/ml

The obtained data for antifungal activity is presented in Table 2; generally, the acetone extract was the most active (MICs 2.50-8.00 μg/ml; MFCs 5-10 μg/ml). *A. fumigatus* and *A. ochraceus* were the most susceptible fungi on the acetone extract, while *T. viride* on the hexane extract (MIC/MFC 1.00/2.50 μg/ml). On the other hand, the methanol extract showed the best activity against *A. niger* (MIC/MFC 6/8 μg/ml), while the water extract was highly active against *A. fumigatus* (MIC/MFC 5/10 μg/ml). The dimethyl sulfoxide extract was the least effective on all the fungi tested (MICs 10-50 μg/ml and MFCs 40-60 μg/ml). Bifonazole showed MICs 100-200 μg/ml and MFCs 200-250 μg/ml, while ketoconazole exhibited MICs 200-2500 μg/ml and MFCs 500-3500 μg/ml. All the extracts were more active than positive controls. Their antifungal potential can be presented as follows: acetone > methanol > hexane > water > dimethyl sulfoxide.

Table 2. In vitro antifungal activity of H. punctata.

Fungi	Acetone extract*	Hexane extract*	Methanol extract*	Water extract*	Dimethyl sulfoxide extract*	Bifonazole*	Ketoconazole*
A. fumigatus	6 9	8 10	6 10	5 10	10 50	150 200	200 500
A. versicolor	5 10	5 10	2.50 5.00	2.50 5.00	20 40	100 200	200 500
A. ochraceus	6 9	8 10	8 10	20 40	50 60	150 200	1500 2000
A. niger	6 9	8 10	6 8	10 10	50 60	150 200	200 500
P. ochrochloron	2.50 5.00	2.50 5.00	2.50 5.00	5 10	20 40	150 200	1000 1000
P. funiculosum	2.50 5.00	2.50 5.00	2.50 5.00	10 20	10 50	200 250	200 500
T. viride	2.50 5.00	1.00 2.50	2.50 5.00	5 10	40 50	200 250	2500 3500
C. albicans	8 9	8 9	5 10	40 50	40 50	100 200	200 300

* MIC/MFC µg/ml

4. Discussion

The bacteria and fungi had not responded identically to the extracts (all displayed higher antifungal activity), which indicated their different modes of action and/or specific metabolic adaptations of the microorganisms. Although it is well known that Gram (-) bacteria are more resistant than Gram (+) ones [23], both the acetone and hexane extracts seem to be gold mines of antimicrobials against them (particularly, their more lipophilic components).

5. Conclusion

Due to their inefficiency, a large variety of antibiotics and mycotics are used to control infections and diseases in animals. These may cause severe hypersensitivity reactions and lead to the resistance of pathogens. Additionally, there is an increasing legislation against the use of synthetic antimicrobial agents. Therefore, natural products for the treatment of bacterial and fungal infections are in the focus. The ultimate goal of the present study is developing effective and inexpensive antimicrobial feed supplements. Since its extracts have shown to be potent bacterial and mold inhibitors, the freshwater bryozoan *H. punctata* can be considered as a promising resource of these agents.

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