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Evaluation of Antimicrobial Potential of the Marine Cyanobacterium, *Rivularia mesenterica*

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Authors' contributions

This work was carried out in collaboration among all authors. Author MS designed the study, performed the statistical analysis, wrote the protocol and wrote the first draft of the manuscript. Authors SL and ZR managed the analyses of the study. Author SL managed the literature searches. All authors read and approved the final manuscript.

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ABSTRACT

Background: Antibiotic resistance is becoming a pivotal concern for public health accelerating the search for new antimicrobial molecules from nature. The prevention and treatment of infectious diseases by applying products from marine organisms, especially Cyanobacteria as a potential and promising source of antimicrobial agents appears as a possible alternative.
Aims: To evaluate the *in vitro* antimicrobial potential of different extracts derived from marine cyanobacterium *Rivularia mesenterica* against Gram-positive and Gram-negative bacteria, including multidrug resistant bacteria, by comparison with clinically relevant antibiotics.
Methodology: The secondary metabolites were extracted from fresh and dried cyanobacterial biomass in water and different organic solvents. Antimicrobial efficacy of different extracts was evaluated by the disc diffusion assay. Additionally, the minimum inhibitory concentrations (MIC) of the ethanol extracts obtained from fresh and dried biomass was also determined.
Results: The ethanol extracts obtained from fresh and dried biomass of *R. mesenterica* showed significant antimicrobial activity against five Gram-positive and five antibiotic resistant Gram-

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negative bacteria and four fungal strains in comparison with the clinically relevant antibiotics. The inhibitory effect of the ethanol extracts was observed, with MIC values in the range 0.06 to 32.00 µg/ml against tested strains. Furthermore, the water extract was inactive against of the tested bacteria and fungi.

Conclusion: These results suggest that the ethanol extracts of *R. mesenterica* possess potent broad spectrum of antimicrobial activity, which can serve as an interesting source for antimicrobial compounds and promising alternative to synthetic antimicrobial drugs discovery.

Keywords: Cyanobacteria; Rivularia mesenterica; extracts; antimicrobial activity; minimum inhibitory concentration; MIC; Gram-positive bacteria; Gram-negative bacteria; fungi.

1. INTRODUCTION

The persistence and proliferation of antibiotic resistance in bacterial pathogens represents a considerable public health concern. Multidrug resistant microorganisms persist and spread worldwide, causing clinical failures in the treatment of infections and public health crises. Therefore, in order to effectively prevent and treat serious bacterial infections and to solve the current acute shortage of antibiotics, the discovery and development of novel antibacterial drugs are urgent. The prevention and treatment of these infectious diseases by using products from marine blue-green algae appears as a possible alternative. Hence, the interest in marine organisms, especially Cyanobacteria as a potential and promising source of antimicrobial agents and as templates for synthetic modification has become the topic of ongoing research [1-5].

Cyanobacteria, also known as blue-green algae, are considered as an important group of ancient slow-growing photosynthetic prokaryotes, which are responsible for producing atmospheric oxygen as well as play a pivotal role in generating a surprisingly structurally diverse constituents of secondary metabolites [4]. To survive in a very competitive environment, marine microalgae had to evolve defense strategies, resulting in an enormous diversity of compounds from different metabolic pathways. Most of these metabolites are biologically active molecules and are varying in their bioactive targets as well as mechanisms of action, and chemical structures. Cyanobacteria naturally accumulate functional isoprenoids such as carotenoids, the phytol of chlorophyll and quinones as essential cofactors for photosynthesis [6]. Some cyanobacteria also produce monoterpenes, sesquiterpenes and hopanoids have been to exhibit significant antimicrobial effects against number of the pathogens [7]. Therefore, cyanobacteria are important as a source of the structurally diverse compounds including peptides, polyketides, alkaloids, lipids as well as terpenes that found broad applications in numerous fields such as in medicine, biochemistry, pharmacology, and industry [4,5,8-10].

Bioactive constituents that are isolated from cyanobacteria have been a major inspiration for the development of many of the pharmaceutical drugs. So far, many bioactive constituents have been successfully isolated and employed as clinical drugs such as dolastatin chemo-therapeutic agents, the vancomycin antibiotic, cyclosporine as an immunosuppressant as well as bleomycin [4,8,10]. Based on several literature surveys, cyanobacteria and their secondary metabolites have attracted enormous scientific interest as promising natural source owing to their unique biological properties ranging from antioxidant, antifungal, anti-inflammatory, chemopreventive to antimicrobial activities [10,11].

Therefore, the ability of Cyanobacteria to produce antimicrobial compounds may be noticed not only as a defence against consumers, competitors, fouling organisms and the pathogens but also as a powerful source of the new bioactive compounds from a antimicrobial drugs discovery. Based on several literature surveys, cyanobacterial metabolites have been described as source of promising antimicrobial compounds in view of their unique antimicrobial activity patterns [11-15]. In this context, cyanobacterial metabolites have attracted considerable attention as serve as defensive agents against various the pathogens.

Therefore, in the present study, metabolites extracted from a filamentous, nitrogen-fixing marine cyanobacterium *R. mesenterica* were evaluated in order to obtain their *in vitro* antimicrobial potential. Blue-green *Rivularia mesenterica* Thuret ex Bornet et Flahault, is a

filamentous,nitrogen-fixing, photolithtoautotrophic marine cyanobacterium that forms beautiful, blue-green spherical colonies in a gelatinous sheath. The genus *Rivularia* belongs to the family Rivulariaceae, order Nostocales, widely distributed in the Croatian littoral zone of the Adriatic Sea [16]. *Rivularia* is distinguished from other members of the family by its free-living macroscopic hemispherical gelatinous colonies, usually found attached to rocks in littoral regions (Fig. 1A). It is characterized by the possession of tapered trichomes, a basal heterocystes, false-branching and a thick sheath often coloured brown with scytonemin (Fig. 1B).

However, few reports have focused on antimicrobial activity of *Rivularia* species. Recently, the the spectrum of antimicrobial, antioxidant and antiacetylcholinesterase activities of the marine cyanobacterium *Rivularia mesenterica* was reported by our research group [17]. Additionally, the inhibitory effect of cyanobacterial extracts of *Rivularia biasolettiana* and *Rivularia haematites* on the growth of microalgae and bacteria have been reported [18]. Furthermore, a previous study conducted on *Rivularia* species demonstrated that the diethyl ether and ethyl acetate extract of *Rivularia* species possesses a strong antibacterial activity against *P. mirabilis* and *Acinetobacter* species [19].

Considering the potential of *Rivularia* species and the interest in new antimicrobial natural compounds, the aim of this study is to evaluate the *in vitro* antimicrobial activity of water and extracts obtained with different organic solvents of *Rivularia mesenterica* by disc diffusion assay. Additionally, the minimum inhibitory concentrations (MIC) of ethanol extracts obtained from fresh and dried biomass were also determined against a collection of representative Gram-positive and Gram-negative bacteria, including multidrug resistant bacteria as well as fungal strains by broth microdilution method and compared with relevant antibiotics.

2. MATERIALS AND METHODS

2.1 Collection and Description of Cyanobacteria

The epilithic free-living macroscopic colonies of *R. mesenterica* were collected on the stone surfaces in the littoral zone of the central Adriatic Sea (Dalmatia) near Split, Croatia. Morphology was studied with a Zeiss Axioplan-2 optical light microscope (Carl Zeiss, Jena, Germany). Taxonomic identification was performed on morphological and cytological characteristics such as the presence of heterocysts, hormogonia, and filament hair formation, using the classification system devised by Rippka et al. [20]. This isolated strain is kept in the culture collection of the Department of Biology, Faculty of Science, University of Split, Croatia, under the number FNSS-219-17.

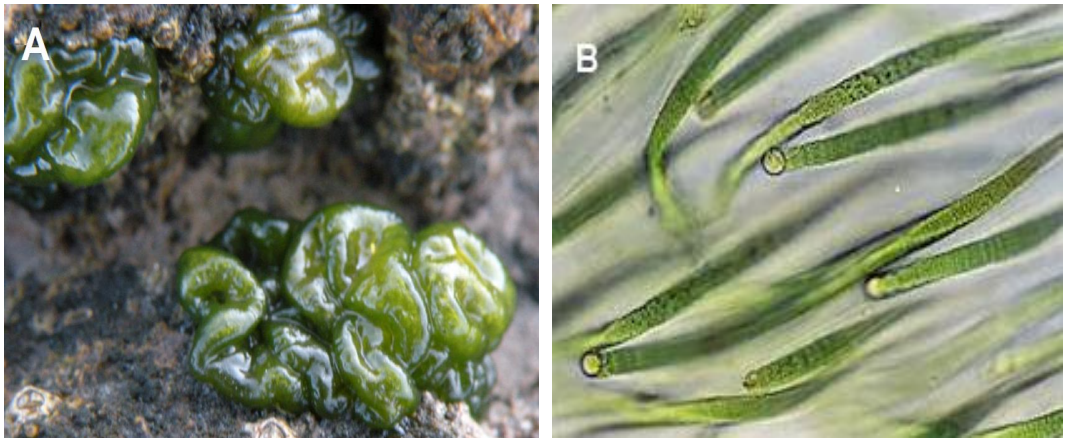

Fig. 1. Morphological characteristics of *Rivularia mesenterica*; A- macroscopic hemispherical gelatinous colonies from stones in littoral zone of the Adriatic Sea (Croatia); B- cytological characteristics- trichomes with basal heterocysts

2.2 Preparation Extracts of Cyanobacterial Biomass

Gelatinous macroscopic colonies of *R. mesenterica* were washed three times with sterilized seawater and once with phosphate-buffered saline (PBS) and then air-dried for two weeks. The air-dried biomass of *R. mesenterica* was pulverized into powdered form. The dried powder (10 g) was extracted with different solvents including distilled water, pentane, methanol and ethanol separately for 2 h at room temperature, filtered through 0.45 µm pore membrane (Pall Life Sciences**,** Ann Arbor, USA) and then concentrated under vacuum to dryness at a temperature between 50 and 60°C. The obtained residue was dissolved in distilled water, pentane, methanol and ethanol separately to give a final concentration of 10 mg/ml of the extract. Cellular water and ethanol extracts were also prepared from fresh biomass. Twenty grams of fresh biomass was mixed with 50 ml of PBS and then homogenized. After centrifugation at 8000 rpm for 5 min, supernatant was collected, followed by filtration to obtain the water-soluble and ethanol extracts. The water and ethanol extracts were freed from solvent by evaporation under vacuum to give a final concentration of 10 mg/ml of the extract. All solvents were purchased from Merck (Darmstadt, Germany).

2.3 *In vitro* Screening Antimicrobial Activities

The different methods were employed for screening of *in vitro* antimicrobial activity. Water and different organic extracts extracted from *R. mesenterica* were screened for their possible antimicrobial activities using agar disc diffusion assay. The minimum inhibitory concentrations (MIC) of the ethanol extracts obtained from fresh and dried biomass were also determined against Gram-positive and Gram-negative bacteria, including multidrug resistant bacteria by broth microdilution method and compared with relevant antibiotics.

2.4 Microbial Strains and Culture Conditions

All the extracts were evaluated for their *in vitro* antibacterial activity. The tested microorganisms were obtained from the culture collection at the American Type Culture Collection (ATCC, Rockville, MD, USA) and at the Microbiology laboratory, Department of Biology, Faculty of Natural Science, University of Split, Croatia (FNSST). The assayed collection included five Gram-positive bacteria *Bacillus cereus* (ATTC 11778), *Enterococcus faecalis* (ATCC 29212), *Micrococcus luteus* (FNSST 0391), *Staphylococcus aureus* (ATCC 25923) *Clostridium perfringens* (FNSST 4999) and five Gram-negative multidrug resistant bacterial strains *Escherichia coli* (FNSST 982), *Klebsiella pneumoniae* (FNSST 011), *Enterobacter cloacae* (FNSST 326), *Pseudomonas aeruginosa* (FNSST 982) and *Chronobacter sakazakii* (FNSST 014). Bacterial strains were cultured overnight at 37°C in tryptic soy broth (TSB) to achieve optical densities corresponding to 10^6 colony forming units. Antifungal activity was assessed on the yeasts strains *Candida albicans* (ATCC 10231), *Saccharomyces cerevisiae* (FNSST 3728) and filamentous fungal strains *Penicillium* sp. (FNSST 724) Stock cultures were maintained at 4°C on slopes of Tryptic soy broth (TSB, Becton Dickinson, Sparks, MD, USA). Active cultures for experiments were prepared by transferring a loopful of cells from the stock cultures to flasks of Mueller–Hinton broth (MHB; Becton Dickinson) for bacteria and Sabouraud dextrose broth (SDB; Becton Dickinson) for fungi that were incubated without agitation for 24 h at 25°C. The cultures were diluted with Sabouraud dextrose broth to achieve optical densities corresponding to 10^4 colony forming units.

2.5 Growth Inhibition by Disc Diffusion Assay

Preliminary antimicrobial screening of the water extract and extracts obtained with different organic solvents was performed using the disc diffusion assay according to CLSI procedure as previously described [21]. Briefly, 100 µl of suspension containing 10^6 colony-forming units (cfu/ml) of bacterial cells was spread on a Mueller Hinton agar (Becton Dickinson, Sparks, MD, USA). The stock solutions of extracts were prepared by dissolving the cyanobacterial material in solvents to a final concentration of 10 mg/ml. The sterile filter discs (6 mm) were individually loaded with 25 µl and 50 µl of stock solution from fresh and dried extracts equivalent to final concentrations at 250 and 500 µg/disc and then placed on the nutrient agar that had been previously inoculated with the target microbial strains. Additionally, solvent employed to dissolve the samples was used as a negative control, and discs containing ampicillin (30 µg) and tetracycline (30 µg), was used as positive controls. Amphotericin B (10 µg) was used as a positive control for fungal strains. All discs were purchased from BBL (Becton Dickinson). The

plates were incubated for overnight at 37°C for bacterial strains and 48 h at 28°C for fungal strains. Antimicrobial activity was assessed by measuring the diameter of the inhibition zone in millimetres, including disc diameter for the test isolates, compared to the controls. Samples were assayed in triplicate for each condition and the diameter of inhibition zones were presented as mean ±SE values.

2.6 Microdilution Assay

The minimal inhibitory concentrations (MIC) of the extracts were determined by the broth microdilution assay via slight modifications to literature procedures [22]. All tests were performed using MHB for bacterial and SDB for fungal strains. Bacterial strains were grown to exponential phase in nutrient broth at 37°C for 18 h and adjusted to a final density of 10^6 cfu/ml by diluting fresh cultures and comparison to McFarland density. A serial doubling dilution of extracts was prepared in a 96-well microtiter plate with MHB and SDB over a range fom 0.06 µg/ml to 500 µg/ml. Aliquot of 5 µl of the inoculum was added into each well. Plates were incubated under normal atmospheric conditions for 24 h at 37°C for bacteria and 48 h at 28°C for fungi, with shaking at 200 rpm. As an indicator of bacterial growth, 50 µl of 0.2 mg/ml p-iodonitrotetrazoliumchloride (INT; Sigma) was added to the wells and incubated at 37 °C for 30 min. The lowest concentration of compound showing no growth was taken as its minimal inhibitory concentration (MIC). The colourless tetrazolium salt acts as an electron acceptor and is reduced to a red-coloured formazan product by biologically active organisms. Where bacterial growth was inhibited, the solution in the well remained clear after incubation with INT. Ampicillin and tetracycline were used in the antibacterial and amphotericin B in the antifungal assay, as positive controls.

3. RESULTS

3.1 Antimicrobial Activity

Preliminary studies were performed to evaluate the *in-vitro* antibacterial efficacy of the water extract and extracts obtained with different organic solvents of *R. mesenterica* against antibiotic susceptible Gram-positive and resistant Gram-negative bacteria as well as fungi by disc diffusion assay. Antibacterial and antifungal potential of different extracts were assessed in terms of the inhibition zone diameter of the microbial growth. As shown in Table 1. the results indicate that the ethanol and methanol extracts of the *R. mesenterica* demonstrated the most potent and broad-spectrum activity against selected clinically important Gram-positive and antibiotic-resistant Gram-negative bacteria, as well as fungi with varying degrees of inhibition under that condition. The mean inhibition diameters of the ethanol extracts were found in the range from 12.1± 1.3 to 25.7± 0.9 mm. Lower activities was observed for extract made from methanol (9.3± 0.7- 21.3± 0.61 mm). On the other hand, the pentane extract was active only against *Micrococcus luteus* and *Candida albicans*. Furthermore, the water extracts were found to be virtually inactive in inhibition of microbial strains, in contrary, growth promotion was observed in most assays. Organic extracts were found to be more active than water extracts. In the present study, the ethanol extracts were found to be a more suitable solvent for the maximum extraction of active metabolites than methanol. Ethanol extracts were found to have variable inhibitory patterns against the Gram-positive and Gram-negative bacteria with maximum inhibition of growth on *Escherichia coli* followed by *Enterobacter sakazakii*. Therefore, ethanol extract was selected for further antimicrobial screening.

Additionally, in order to compare *in vitro* effects of the ethanol extracts obtained from fresh and dried biomass of *R. mesenterica* on the inhibition the growth of tested strains the disc diffusion assays were performed. Activities of the ethanol extracts were expressed as the mean diameter of the growth inhibition zone (mm) against these microorganisms and are listed in Table 2, along with the activity of the reference compounds ampicillin and tetracycline. It can be seen that both extracts demonstrated potent and broad spectrum activities against selected clinically important pathogens with varying degrees of inhibition under that condition.

Generally, the ethanol extract from dried biomass showed better antimicrobial effect against Gram-positive bacteria, with inhibition zones diameter were found in the ranged from 21.7 ± 0.7 to 32.2 ± 0.4 mm at concentration on the 500 µg per disc. It was also worth noting that ethanol extracts from fresh biomass also displayed less potent inhibitory effects against Gram-negative bacteria, with inhibition zones ranged from 20.2 ± 0.4 to 26.9 ± 0.8 mm. Interestingly, the ethanol extracts obtained from both, fresh and dried biomass of *R. mesenterica*, showed the most potent antifungal activities.

3.2 Determination of Minimum Inhibitory Concentration (MIC)

In order to confirm the found antimicrobial efficacy, the ethanol extracts obtained from fresh and dried biomass *of R. mesenterica* were then tested for activity against clinically relevant pathogens to determine MIC values by a broth microdilution method. The antimicrobial bioassay results of the target extracts and the reference compounds ampicillin and tetracycline are summarized in Table 3. The activity was represented in terms of minimum inhibitory concentration (MIC) and defined as the minimum concentration required inhibiting bacterial growth. The inhibitory effect of the ethanol extracts was observed, with MIC values in the range from 0.06 to 32.00 µg/ml against of the tested strains. Ethanol extract obtained from fresh biomass displayed more potent antimicrobial activity with a MIC values from 0.06 to 16.00 µg/ml as compared with the extracts made from dried biomass (MIC= 0.06-32.00 µg/ml).

It is interesting to note that extracts made from dried biomass exhibited excellent *in vitro* activity against a broad spectrum of clinically important Gram-positive pathogens such as *Staphylococcus aureus* (MIC = 0.12 µg/ml) and *Enterococcus faecalis* (MIC = 0.06 µg/ml) which is 8 to 32-fold better than that the tetracycline. Data indicated that Gram-positive food pathogen *Clostridium perfringens* showed resistance to the ethanol extract from dried biomass as compared with other strains tested, with a MIC of 32.00 µg/ml, which was 64-fold less effective than the values obtained for standard drugs ampicillin and tetracycline.

It is particularly important that both ethanol extracts of *R. mesenterica* demonstrated promising activity against *Pseudomonas aeruginosa* which is one of the most prevalent multidrug-resistant pathogen worldwide with equal MIC of 0.06 µg/ml, which was 1042-fold more potent than the ampicillin and 553-fold more active than the tetracycline.

Furthermore, extracts of *R. mesenterica* exhibited excellent *in vitro* activity against a broad spectrum of clinically important multidrug resistant Gram-negative pathogens such as *Escherichia coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae* at MIC values of 0.12 µg/ml, which is 133 to 1066-fold more superior antimicrobial activity than the values obtained for standard drug tetracycline.

Table 1. Inhibitory effects of different extracts (ethanol, methanol, pentane and water) of *R. mesenterica*, determined by the disc diffusion assay

Test strains	Diameters of the inhibition zones (mm)*			
	Ethanol	Methanol	Pentane	Water
Gram-positive bacteria				
Bacillus cereus	18.4 ± 1.1	12.7± 0.5	na	na
Clostridium perfringens	11.2 ± 0.8	9.3 ± 0.7	na	na
Micrococcus luteus	25.3 ± 1.4	12.2 ± 1.7	8.6 ± 1.3	na
Staphylococcus aureus	17.9 ± 0.3	14.7 ± 0.9	na	na
Gram-negative bacteria				
Enterobacter sakazakii	25.2 ± 0.7	21.3 ± 0.6	na	na
Escherichia coli	25.7 ± 0.9	16.2 ± 1.1	na	na
Enterobacter cloacae	18.1 ± 0.4	12.0 ± 0.6	na	na
Klebsiella pneumoniae	12.5 ± 0.5	10.3 ± 0.5	na	na
Pseudomonas aeruginosa	17.9 ± 1.2	14.6 ± 0.9	na	na
Fungi				
Aspergillus niger	16.0 ± 0.7	16.2 ± 1.4	na	na
Candida albicans	14.4 ± 1.5	14.6 ± 0.5	8.5 ± 0.4	na
Penicillium sp.	12.1 ± 1.3	14.2 ± 0.9	na	na
Saccharomyces cerevisiae	21.8 ± 0.2	20.9 ± 0.6	na	na

**Data are expressed as mean ± S.D. (n = 3); na = No activity*

Table 2. Inhibitory effects of *R. mesenterica* ethanol extracts from fresh and dried biomass against selected strains as determined by the disc diffusion method in comparison with standard antibiotics

Test strains	Diameters of the inhibition zones (mm)[a]					
	Fresh extracts[c]		Dried extracts[c]		Standard antibiotics[b,]	
	500 µg	250 µg	500 µg	250 µg	Amp	Tet
Gram-positive bacteria						
Bacillus cereus	20.4 ± 2.1	18.6 ± 0.2	21.7 ± 0.7	19.1 ± 1.7	19.4	19.2
Clostridium perfriigens	27.3 ± 1.8	25.1 ± 0.9	32.2 ± 0.4	24.7 ± 0.5	12.7	26.4
Enterococcus faecalis	28.1 ± 1.9	26.9 ± 1.2	30.4 ± 0.5	28.6 ± 0.3	15.3	12.3
Micrococcus luteus	25.5 ± 1.7	23.2 ± 0.3	24.9 ± 0.9	22.3 ± 1.1	14.1	12.6
Staphylococcus aureus	26.9 ± 0,9	23.6 ± 0.8	24.2 ± 1.3	22.3 ± 0.4	12.5	14.9
Gram-negative bacteria						
Enterobacter sakazakii	25.4 ± 2.2	21.1 ± 1.2	22.3 ± 1.1	19.0 ± 0.9	6.6	14.5
Escherichia coli	25.7 ± 1.3	23.2 ± 0.4	22.7 ± 0.7	20.7 ± 1.8	6.0	8.6
Enterobacter cloacae	26.9 ± 0.8	24.5 ± 0.9	24.3 ± 1.2	22.0 ± 0.6	6.0	8.5
Klebsiella pneumoniae	25.3 ± 1.1	24.7 ± 1.6	24.4 ± 0.7	22.5 ± 0.4	6.0	6.9
Pseudomonas aeruginosa	20.2 ± 0.4	18.2 ±0.6.	22.6 ± 0.2	20.6± 0.2	6.0	10.2
Fungi					Amphotericin B	
Aspergillus niger	30.9 ± 0.6	29.8 ± 0.4	32.2 ± 0.8	28.4 ± 1.8	21.6	
Candida albicans	35.6 ± 2.1	30.3 ± 2.1	35.9 ± 1.7	30.6 ± 0.4	24.3	
Penicillium sp.	34.2 ± 1.9	30.8 ± 0.7	30.2 ± 0.9	25.0 ± 0.9	21.5	
Saccharomyces cerevisiae	31.4 ± 1.1	30.2 ± 2.1	30.1± 1.3	24.3 ± 1.6	19.4	

[a]*Diameter of inhibition zone (values in mm) around the disc: 250 and 500 µg/disc.*
[b]*Standard antibiotics disc: Amp-ampicillin (30 µg); Tet - tetracycline (30 µg); Amphotericin B (10-µg).*
[c]*Values are expressed as mean ± S.D. (n = 3).*

Table 3. Minimum inhibitory concentrations of *R. mesenterica* ethanol extracts from fresh and dried biomass against selected strains as determined by the broth microdilution method in comparison with standard antibiotics (MIC in µg/ml)

Test strains	Minimum inhibitory concentrations (MIC) µg/ml			
	Fresh extracts	Dried extracts	Standard antibiotic	
			Amp	Tet
Gram- positive bacteria				
Bacillus cereus	0.50	0.50	1.00	2.00
Clostridium perfringens	4.00	32.00	0.50	0.50
Enterococcus faecalis	2.00	0.06	1.00	2.00
Micrococcus luteus	4.00	0.12	0.50	2.00
Staphylococcus aureus	0.50	0.12	0.20	1.00
Gram- negative bacteria				
Enterobacter sakazakii	4.00	4.00	64.0	8.00
Escherichia coli	0.12	0.12	250.0	32.0
Enterobacter cloacae	0.12	0.12	64.0	16.0
Klebsiella pneumoniae	0.12	0.12	125.0	128.0
Pseudomonas aeruginosa	0.12	0.12	125.0	64.0
Fungi			Amphotericin B	
Aspergillus niger	0.12	1.00	3.12	
Candida albicans	4.00	4.00	6.25	
Penicillium sp.	1.00	8.00	3.12	
Saccharomyces cerevisiae	0.06	8.00	6.25	

The results of the antifungal screening showed that ethanol extracts from fresh and dried biomass were found to possess strong antifungal effects against all of the fungal strains tested, in range from 0.12 to 8.0 µg/ml. The both ethanol extracts exhibited the most promising antifungal efficacy against *Aspergillus niger,* with MIC values of 0.12 and 1.00 *µ*g/ml, respectively, even stronger than amphotericin-B (MIC = 3.12 *µ*g/ml). The highest antifungal activity was observed for the ethanol extracts obtained from fresh biomass of *R. mesenterica* against *Saccharomyces cerevisiae* (MIC= 0.06 µg/mL) *and Penicillium sp.* (MIC = 1.00 µg/ml), which were more effective than amphotericin-B (MIC = 6.25 and 3.12 *µ*g/mL). However, the ethanol extracts obtained from dried biomass displayed less and equipotent antifungal efficacy against *Saccharomyces cerevisiae and Penicillium sp.* with an MIC value of 8.00 µg/ml, compared to standard drugs, amphotericin-B (MIC = 3.12 and 6.25 *µ*g/ml, respectively). However, the data also showed that the ethanol extract obtained from dried biomass of *R. mesenterica,* after drying process, retain their antimicrobial potential.

4. DISCUSSION

Infectious diseases are the second leading cause of death worldwide and cause significant morbidity, having a profound effect on global health. The emergence of multidrug resistant bacteria has become a serious threat to public health and is considered one of the greatest challenges for contemporary medicine. In this context, cyanobacterial metabolites have attracted considerable attention as defensive agents against various pathogens. Based on several literature surveys, cyanobacterial metabolites have been described as promising source of antimicrobial compounds in view of their unique bioactivity patterns [23-27].

Previous studies have shown the promising antibacterial and antifungal activities of secondary metabolites of cyanobacteria, extracted from a variety of taxa and geographic origins such as: *Spirulina platensis*, *Synechocystis* and *Synechococcus*, *Calothrix parietina*, *Oscillatoria redekei*, *Nostoc* sp, *Nostoc muscorum*, *Scytonema hofmanni*, *Fischerella* and *Phormidium* [13-15, 23-27]. They have also reported that the extracts obtained with different solvents were effective against both Gram-positive and Gram-negative bacteria.

In the present study, results revealed that both of ethanol and methanol extracts from *R. mesenterica* have demonstrated most potent and broad-spectrum activity against selected clinically important Gram-positive and antibiotic-resistant Gram-negative bacteria, as well as fungi. On the other hand, the pentane extract exhibited activity only against *Micrococcus luteus* and *Candida albicans*. However, the water extract of *R. mesenterica* exhibited no antimicrobial activity against any of the bacterial or fungal isolates tested as compared with the other organic extracts. The present results are in agreement with those of Malathi et al. [7], who showed that aqueous extracts are generally less potent in their bioactivity than organic extracts. The reasons for this could be that all of the components from *R. mesenterica* active against microorganisms, aromatic or saturated organic compounds, are most often obtained through ethanol or methanol extraction. Significant differences in the antimicrobial activity of extracts from *R. mesenterica* depending on the solvent used for the extraction could be due to the presence of different bioactive compounds responsible for their inhibitory effects, which are found to be in accordance with previous studies [25-28].

According to the results of the present study, the ethanol extracts from fresh and dried biomass of *R. mesenterica* possesses a potent and broad-spectrum activity not only against Gram-positive bacteria, but also demonstrated significant antibacterial effects against multidrug resistant Gram-negative bacteria, with MIC values in the range from 0.06 to 32.00 µg/ml.

These results revealed that ethanol extract made from dried biomass exhibited excellent *in vitro* activity against a broad spectrum of clinically important Gram-positive pathogens such as *Staphylococcus aureus* (MIC = 0.12 µg/ml) and *Enterococcus faecalis* (MIC = 0.06 µg/ml), which was 8 to 32-fold better antimicrobial activity than the tetracycline.

Especially, the growth of *P. aeruginosa* was remarkably inhibited by the ethanol extract of *R. mesenterica* (MIC 0.06 µg/ml), which was 1042-fold more potent than the ampicillin and 553-fold more active than the tetracycline. It seems very likely, therefore, that the antibacterial compounds extracted from *R. mesenterica* may inhibit bacteria by a different mechanism than that of currently used antibiotics, which may provide

new means of studying the mechanisms of bacterial control at a molecular level.

Previous studies have also reported that a variety of cyanobacteria produce natural products that exhibit antifungal activity [26- 28]. According to our results, the ethanol extracts of *R. mesenterica* affected the growth of different fungi, with MIC values in the range from 0.12 to 8.0 µg/ml, suggest that the compounds present in the extracts could play an active role in the protection against fungi related to several diseases. The highest antifungal activity was observed for the ethanol extracts obtained from fresh biomass of *R. mesenterica* against *Saccharomyces cerevisiae* (MIC= 0.06 µg/mL) *and Penicillium sp.* (MIC = 1.00 µg/ml) and *Candida albicans* (MIC = 4.00 µg/ml), which was superior to amphotericin-B. These results are consistent with previous studies of antifungal effects of extracts obtained from *Nostoc muscorum* and *Phormidium* were also found to possess promising antifungal properties [27,28].

In previous screening studies, it has been reported that antimicrobial activities of cyanobacterial secondary metabolites were more effective against Gram-positive than Gram-negative bacteria [12, 23]. The tolerance of Gram-negative bacteria to metabolite of cyanobacteria has been described to have the presence of a hydrophilic outer membrane that blocks the penetration of hydrophobic metabolite into target cell membrane.

Furthermore, the data of the present study has shown that the extract of *R. mesenterica* significantly inhibited the growth of several Gram-negative bacteria which were highly resistant to antibiotics such as *Escherichia coli*, *Enterobacter cloacae* and *Klebsiella pneumoniae* at MIC values of 0.12 µg/ml. Data have indicated that extracts possess the ability to disrupt the membranes of Gram-negative bacteria which may significantly increase diffusion rate of the extracts into the cell thus, increasing their efficacy. Outer membrane permeability for antibacterial compounds largely depends on the size, polarity and lipophilicity of compounds, which influence the efficiency with antimicrobials diffuse over the outer membrane. Moreover, despite the inhibitory effects against multidrug resistant Gram-negative bacteria, the ethanol extract may have molecules capable to restore the efficacy of antibiotics in multidrug resistant Gram-negative by increasing the diffusion of antibiotics through the bacterial membrane, but these effects will still be investigated.

The present results, when compared with the data previously obtained [24-26], showed higher antimicrobial potential, especially on the Gram-negative bacteria. The results obtained in this investigation suggest that ethanol extracts of *R. mesenterica* have potent activity capable of inhibiting the growth not only susceptible but also multidrug resistant bacteria, as well as fungi.

5. CONCLUSION

Our results reinforce the concept of exploiting cyanobacteria as viable producers of secondary metabolites, by demonstrating their biological potential with a novel platform for the development of new drugs. The present study is the first screening *in vitro* bioactivity of marine cyanobacterium *R. mesenterica,* which could be directly relevant for prophylaxis and therapy of infections associated with the tested microorganisms. Therefore, *R. mesenterica* is interesting source for biologically active compounds, which may be a promising alternative to synthetic substances. As it is a natural valuable source with antimicrobial activities. The cyanobacterium will be subjected for further research with the objective of isolating and identifying the bioactive compounds responsible for the activities demonstrated in this report. This investigation has served as the basis for further identification and studies on active compounds. In a near future, we plan to carry out pharmacological and toxicological studies of this cyanobacterium and to investigate the antimicrobial mechanisms of action.

Furthermore, the encouraging biological activities seen in this study show that the Croatian coastline is a potential source of cyanobacterial species worthy of further investigation.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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