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Original Research Article

The antiproliferative and antimicrobial effects of cultivated *Anabaena circinalis* Rabenhorts ex Bornet and Flahault and *Nostoc entophytum* Bornet and Flahault

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Abstract

Purpose: *To investigate the antiproliferative and antimicrobial effects of cultivated Anabaena circinalis Rabenhorst Ex Bornet and Flahault [Synonym: Dolichospermum sigmoideum (Nygaard) Wacklin, L. Hoffmann and Komarek) and Nostoc entophytum Bornet and Flahault (Synonym: Nostoc paludosum Kützing ex Bornet and Flahault).*
Methods: *The algae extracts were prepared using 0.5 M Tris-HCL pH: 8.00, N-butanol, Ethanol and Dimethyl sulfoxide, and then tested on Staphylococcus aureus ATCC 25923, Bacillus subtilis ATCC 6633, Listeria monocytogenes ATCC 7644, Escherichia coli O 157:H7, Pseudomonas aeruginosa ATCC 27853, Salmonella typhimurium CCM 5445, Candida albicans ATCC 10239 using the disc diffusion method in order to determine their antimicrobial effects. The anti-cancer activities of these two algae were tested against cancerous cell lines using BrdU cell proliferation ELISA method. The inhibition of these algae ethanol and butanol extracts were tested against Vero and HeLa carcinoma cells in concentrations of 100, 250 and 500 µg/mL.*
Results: *Anabaena circinalis (AC) and Nostoc entophytum (NE) antimicrobial properties against the tes organisms. The buffer extract obtained from AC showed the highest level of antimicrobial activity against L. monocytogenes ATCC 7644 while the buffer extract from NE displayed the highest antimicrobial activity against E. coli O 157:H7. The anti-proliferative data indicate that NE has effective anti-cancer properties. Furthermore, the results showed that cyanobacteria species were superior to DMSO and control groups in terms of anti-cancer activity in tumor cells. NE exhibited significant ($p < 0.05$) anti-proliferative effects at all three concentrations (100, 250, 500 µg/mL), compared to DMSO while AC exhibited significant anti-proliferative activity only at 500 µg/mL concentration ($p < 0.01$).*
Conclusion: *The results indicate that extracts possess antimicrobial and antiproliferative antivities. However, further studies are required to ascertain their clinical efficacy.*

Keywords: *Anabaena circinalis, Nostoc entophytum, Cyanobacteria, Antiproliferative, Antimicrobial*

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INTRODUCTION

Algae are photosynthetic organisms living in all aquatic habitats on Earth. They may be prokaryotic or eukaryotic in terms of organization level. Cyanobacteria, also known as blue-green algae, are prokaryotic, and are also able to endure pretty extreme conditions. Secondary

metabolites obtained from these algae have important properties. In recent years, the interest in biological activities of these molecules obtained from cyanobacteria has increased [1,2]. In addition, cyanobacterial secondary metabolites have been shown to have hypocholesterolemic, enzyme inhibiting, and other pharmacological effects. These natural products are not only used as raw drug material, but also as structural models in the production of synthetic molecules [3].

Phycocyanin pigment in cyanobacteria has been found to have anti-cancer and immune booster effects. Oral administration of spirulina has been found to lead to a decrease in cancer cells, and a boost in immune system. Moreover, C-phycocyanin (C-PC) found in cyanobacteria has been found to influence growth. New compounds with polypeptide structures obtained from filament cyanobacteria are studied as part of antiproliferative drug development [4].

The aim of this was to explore antimicrobial and antiproliferative effects of *Anabaena circinalis* (AC) and *Nostoc entophytum* (NE) both of which are cyanobacteria with a filament structure.

EXPERIMENTAL

Isolation and cultivation of Cyanobacteria

Algae samples were taken from benthic and pelagic habitats at Yeşilırmak River, Tokat (Turkey) and brought in plastic containers to the Microalgae Cultivation Laboratory, GOU. The samples were filtered through Whatman GF/A filter paper using a water tromp. After examination under a light microscope, some species were taken to liquid culture after being selected under an inverted microscope using the microinjection method. Algae were grown in Allen, BG11, and F2 liquid culture media in a Sanyo MLR 351 climatic chamber at (155 µmol/m^2/s, Light: Dark period) at 26 ^oC, under appropriate growth conditions [5,6].

Antimicrobial assay

Cyanobacteria extraction and impregnation of extracts into discs

Cyanobacteria species were prepared for extraction by being crushed with liquid nitrogen. Extraction solvents (N-butanol and Ethanol) were applied to the samples in order to obtain extracts. 20 µL of each extract was infused into 6 mm sterile antibiotic discs and then dried over 24 hours [7].

Microorganism strains

For antimicrobial tests, 7 microorganism strains (*Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633, *Listeria monocytogenes* ATCC 7644, *Escherichia coli* O157:H7, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella typhimurium* CCM 5445, and *Candida albicans* ATCC 10239) were selected and obtained from the Culture Collection of Biotechnology Laboratory at Gazi University. Solid and liquid media (nutrient agar, nutrient broth, malt extract agar, malt extract broth) were used to grow test microorganisms [8].

Standardization of test microorganisms

Test microorganism cultures were compared with the 0.5 McFarland turbidity standards for standardization prior to applying the disc diffusion method [9].

Disc diffusion method

For microorganism inoculation into the media, a sterile swab was dipped in the microorganism suspension and applied to all parts of the nutrient agar and malt extract agar media. The bacteria were incubated at 37 °C over 24 hours, whilst the *Candida albicans* was incubated at 30°C over 48 hours. Trials were carried out under aseptic conditions with 3 parallel. Inhibition zones formed at the end of incubation period were measured using calipers, with zone diameters being recorded in terms of mm (millimeters) [7].

Control group

Standard antibiotic discs (Penicillin, ampicillin, gentamicin, chloramphenicol, streptomycin and nystatin) (i2a, Perols Cedex, France) were used for positive control, against solvent-soaked (0.5 M Tris-HCL pH:8.00, n-butanol and ethanol) discs. Furthermore, empty antibiotic discs were used for negative control.

Determination of antiproliferative activity

The antiproliferative activities of these were applied to cancerous cell lines using the BrdU cell proliferation ELISA method. Vero (African green monkey kidney cells) and HeLa (Human uterus carcinoma cells) were used as cell lines.

Cells prepared with DMEM medium were incubated with trypsin-EDTA (10 mL) in a CO_2 incubator at 37°C for 1-2 min, whereupon the cells attached to the surface were then removed. 10 mL DMEM (Dulbecco's Modified Eagle's medium) was added to the flask that was

removed from the incubator in order to neutralize the medium. The flask was thoroughly shaken, cell suspension was transferred into a falcon tube, and then settled with centrifuge (600 rpm, 5 min.). This process was repeated every four days. During each repetition, cells were checked for growth and contamination under an inverted microscope [10].

Cyanobacterial extracts (AC, NE) in 3 different concentrations (100, 250 and 500 µg/mL) were added to of the wells, except for the control group, and a total liquid volume of 200 µL was reached.

Sterile solvent–DMSO (Dimethyl sülfoxide) was added to the negative control wells instead of test substances, upon which cells were then incubated over a 24hour period. At the end of this period, the results were obtained using the BrdU cell ELISA method in accordance with the protocol of the manufacturer. Absorbance at a 450 and 650 nm wavelength was measured the in ELISA reader [11].

Statistical analysis

SPSS (Statistical Package for the Social Sciences) was used for statistical analysis of any anti-cancer activity. Differences between experimental groups were assessed using the One-way ANOVA and post hoc Duncan tests. $P < 0.01$ was considered to be statistically significant. The results are presented as a mean ± standard error (SE).

RESULTS

Antimicrobial activity

The buffer extract obtained from *Anabaena circinalis* (AC) showed the highest level of antimicrobial activity against *L. monocytogenes* ATCC 7644. *B. subtilis* ATCC 6633 was found to be the most sensitive microorganism against the buffer (AC- *Anabaena circinalis*), n-butanol, and ethanol extracts, whereas *S. typhimurium* CCM 5445 proved to be the most resistant microorganism (Figure 1).

The buffer extract obtained from NE- *Nostoc entophytum* showed the highest antimicrobial activity against *E. coli* O 157:H7. *E. coli* O 157:H7 and *C. albicans* ATCC 10239 showed to be the most sensitive microorganisms against NE- *Nostoc entophytum* buffer, n-butanol, and ethanol extracts, whereas *L. monocytogenes* ATCC 7644 revealed itself as being most resistant microorganism (Figure 2).

Positive control: Carried out to examine standard. Penicillin, ampicillin, gentamicin, chloramphenicol, streptomycin, and nystatin antibiotic discs were used (Figure 3).

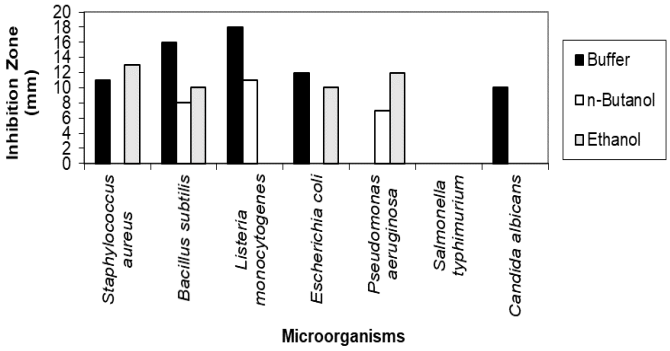

Figure 1: Inhibition zone diameter values of the AC- *Anabaena circinalis* extracts on test microorganisms

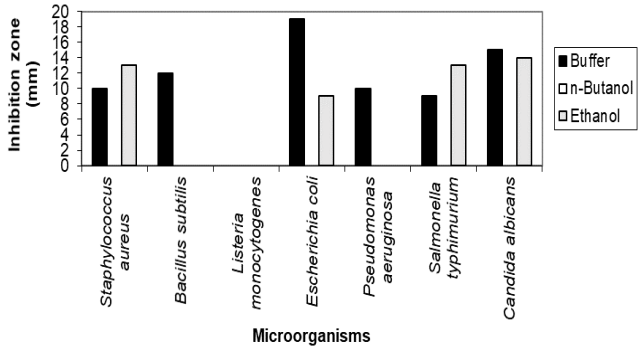

Figure 2: Inhibition zone diameter values of the NE- *Nostoc entophytum* extracts on test microorganisms

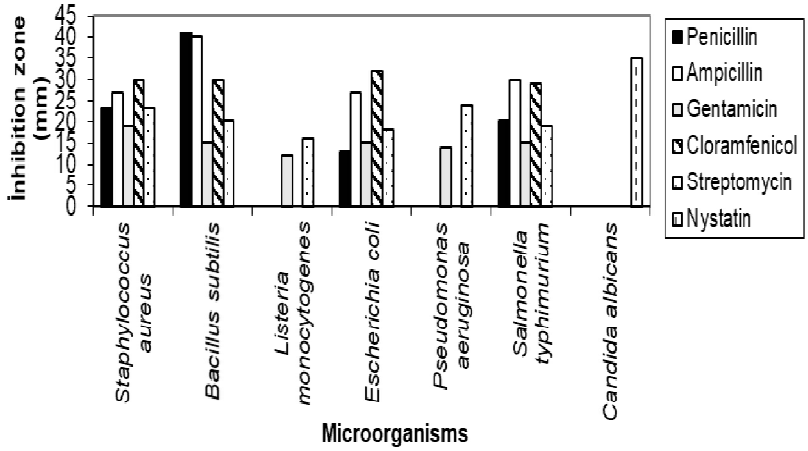

Figure 3: Inhibition zones in positive control

Solvent-soaked discs and empty antibiotic discs were used as negative control. No inhibition zone was observed during the negative control studies. According to these results, there was no contamination stemming from solvents or empty antibiotic discs.

Antiproliferative activity

There were significant differences between groups in terms of test results of the 100, 250 and 500 µg/mL concentrations ($p<0.01$). The multiple comparison test (Duncan) showed that cyanobacteria species (AC, NE) were superior to DMSO and control groups in terms of anti-cancer test results in the afore mentioned concentrations (Table 1).

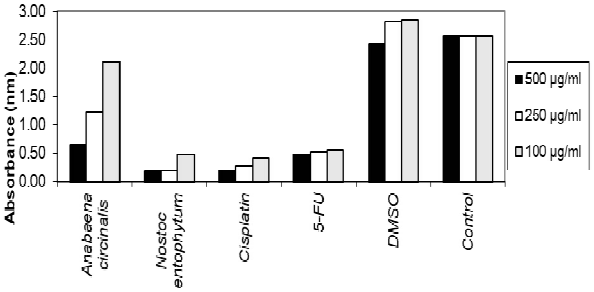

Figure 4: Antiproliferative activity of algae on Vero cells. Cisplatin, anti-cancer compound: 5-FU, anti-cancer compound: dimethyl sulfoxide (DMSO)

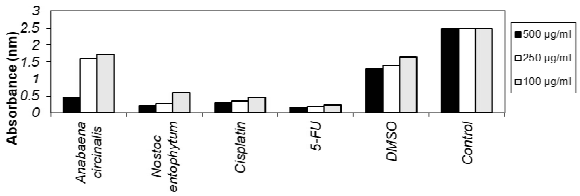

Figure 5: Anti-cancer activity of the extracts in HeLa cells. ***Note:*** cisplatin, anti-cancer compound: 5-FU, anti-cancer compound: dimethyl sulfoxide (DMSO) were used a controls

There were significant differences between groups in terms of the test results of the three concentrations. The multiple comparison test (Duncan) showed that cyanobacteria species were superior to DMSO and control groups in terms of anti-cancer test results in the 500 µg/mL concentration($p<0.01$). The multiple comparison test (Duncan) showed that, among cyanobacteria species, NE was superior to DMSO and control groups in terms of antiproliferative test results of the 100 and 250 µg/mL in concentrations ($p<0.01$) (Table 2, Figure 5).

DISCUSSION

Antimicrobial resistance has become a worldwide problem for both medicine as well as agriculture. Antibiotic-resistant bacteria species seriously threaten animal and human health. Clinical

Table 1: Antiproliferative activity of extracts in Vero cells

	Vero	For 100 µg/mL	For 250 µg/mL	For 500 µg/mL
Group	***Replicate (n)***	***x±(SD)***	***x±(SD)***	***x±(SD)***
Control	3	2.561±0.171	2.561±0.171	2.561±0.171
DMSO	3	2.844±0.040	2.808±0.041	2.427±0.355
Anabaena circinalis	3	2.116±0.042	1.224±0.158	0.644±0.048
Nostoc entophytum	3	0.471±0.142	0.198±0.016	0.178±0.058

Note: *x±(SD = mean ± standard deviation*

Table 2: Anti-cancer activity in HeLa cells

HeLa		For 100 µg/mL	For 250 µg/mL	For 500 µg/mL
Group	***Replicate (n)***	***x±(SD)***	***x±(SD)***	***x±(SD)***
Control	3	2.266±0.023	2.266±0.023	2.266±0.023
DMSO	3	1.630±0.030	1.346±0.045	1.240±0.045
Anabaena circinalis	3	1.663±0.060	1.560±0.036	0.413±0.041
Nostoc entophytum	3	0.543±0.040	0.240±0.036	0.193±0.025

Note: *x±(SD = mean ± standard deviation*

studies on the resistance mechanism has allowed for the defining of clinical uses of all antimicrobials [12]. Knowing how bacterial resistance occurs will help us understand its interaction with antimicrobials, microorganisms, and the environment. A sensitive bacterium becomes a resistant bacteria by developing resistance against antimicrobials through either intrinsic or extrinsic factors. Intrinsic resistant development is related to the existence of resistance genes within the bacteria in question. Extrinsic resistance development may stem from any number of reasons, such as the use of antimicrobials without therapeutic properties, or changes in genomes due to sudden mutations. The use of substances beyond therapeutic dosage, in particular, is one of the most common reasons behind bacteria resistance [13].

In Turkey, various studies have been conducted in order to examine antimicrobial activities of a variety of algae species. Most of these studies focus on various Cyanobacteria and Chlorophyta species such as *Spirulina platensis*, *Chrococcus* sp., *Oscillatoria* sp., *Synechocystis aquatilis, Anabaena* sp., *Oscillatorialimosa*, *O. limnetica* (Synonymous *Pseudoanabaena limnetica*), *Phormidium tenue*, *Chlorella vulgaris,* and *Spirulina major* [14-16].

In the antimicrobial activity section of this study, extracts prepared with the buffer showed the highest degree of activity, whereas extracts prepared with N-butanol showed the lowest level of activity amongst the solvents used. *Anabaena circinalis* and *Nostoc entophytum* were found to be the most effective cyanobacteria species in terms of antimicrobial activity. Sánchez-Saavedra *et al* found that all extracts studied in their study inhibited *Bacillus subtilis* growth [17]. In this study, *Bacillus subtilis* and *Esherichia coli* were found to be sensitive microorganisms. These results are similar to those obtained by Sánchez-Saavedra *et al*. Madhumathi *et al* used acetone, ethanol, and methanol as solvents and, what is more, ethanol extracts were found to show antimicrobial activity against both Gram negative and Gram positive bacteria, which is consistent with our findings [18].

Demiriz *et al* had used methanol, ethanol, n-butanol, acetone, hexane, and 0.5 M *tris*-HCl pH 8.00, and found that the buffer extracts had seen the highest antimicrobial activity against *S. aureus* ATCC 19213, *B. subtilis* ATCC 6633, *S. enteretidis* ATCC 13076, and *E. coli* O157: H7, which is similar to our findings [16]. A number of studies aimed at investigating the antiproliferative properties of cyanobacteria show that the number of species is increasing day by day. Among such studies, Suzuki *et al* studied *Anabaena cylindrica* and *Anabaena variabilis,* Wu *et al* evaluated *Spirulinamaxima*, Dzhambazov *et al* had studied *Phormidium molle*, and Chen and Wong investigated *Spirulina platensis* [19-22]. In addition, Oh *et al* looked at *Spirulinamaxima*, Syahril *et al* studied *Spirulina platensis*, while Shanab *et al* examined *Anabaena flous, A. oryzae, Nostoc humifusum, N. muscorum*, *Oscillatoria* sp., *Spirulina platensis*, *Phormidium fragile* and *Wollea saccate* [23-25].

The number of Turkish studies that have been done on anti-cancer properties of cyanobacteria species is quite limited. Whilst both of the cyanobacteria species tested in our study were found to have antiproliferative activities against cancer cell lines, extracts obtained from NE were found to have a higher level activity when compared to the other species. In one study conducted by Syahril *et al* on *Chlorella vulgaris* and *Spirulina platensis*, MTT assay - a method based on mitochondrial activity - was applied to MCF-7 (human breast cancer cell line), HepG2 (human liver cancer cell line), and WRL-68 (normal cell line) [24].

The authors found that ethanol extract of *S. platensis* did not show any effect over either the 24 or 48-hour treatment periods, however it did show an antiproliferative effect against the human breast cancer cell line MCF-7 over the 72-h treatment period in concentration of 85 µg/mL. In our study, DMSO-dimethyl sulfoxide extracts of *Nostocentophytum* were found to display antiproliferative effects against Vero and HeLa cell lines over a 24-h treatment period at 100, 250 and 500 µg/mL concentrations. The results of this present study suggest that valuable bioactive compounds, as well as the

pharmacological effects of these compounds ought to be investigated further through more comprehensive studies as a service to humanity.

CONCLUSION

The findings of the study indicate that the extracts of the algae species evaluated possess pronounced antimicrobial and antiproliferative activities, However, the bioactive compounds of *Anabaena circinalis* and *Nostoc entophytum* need to be further investigated to ascertain biological properties.

The gastrointestinal polyps and associated symptoms disappeared after approximately 1 year of TCHM therapy without any complications during the follow-up. This case suggests that TCHM could play an important role in the treatment of gastrointestinal polyps. It may be a better choice for the patients who refuse surgery or cannot be surgically operated on, because the Chinese traditional medicine treatment of chronic gastritis and gastrointestinal polyps method is simple and less painful. Relevant data are however limited, and randomized controlled trials are still needed to confirm its efficacy in a larger population.

DECLARATIONS

Acknowledgement

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Conflict of Interest

No conflict of interest associated with this work.

Contribution of Authors

The authors declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by them.

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