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## IN VITRO EVALUATION OF ANTIMICROBIAL AND BIOFILM INHIBITORY ACTIVITY OF *SPIRULINA PLATENSIS* EXTRACTS

**Abstract. Background.** Over recent decades, the incidence of microbial infections has been increasing due to a greater number of immunocompromised people, as well as due to resistant microorganisms involved within their etiology. The increasing resistance of microbial strains to antibiotics, along with the biofilm formation are major problems in the management of infections caused by these organisms. Currently, an impressive number of antimicrobial drugs have been obtained from natural sources. Some cyanobacteria species as *Spirulina platensis*, have the potential to produce a large number of antimicrobial substances and are considered as suitable organisms for being exploited as antimicrobial agents against pathogenic bacteria and fungi. **The purpose of this study** was to determine the antimicrobial and antibiofilm activity of *Spirulina platensis* extracts. Material and methods. Antibacterial activities were determined through minimum inhibitory concentration (MIC), minimum bactericidal/fungicidal concentration (MBC/MFC), and time-killing kinetics. Biofilm formation was detected by microtiter plate method. Statistical analysis involved a one-way analysis of variance. **Results.** The current study proved the *in vitro* antibacterial, antifungal, and antibiofilm activity of *Spirulina platensis* extracts (ES, ES-1 and ES-2). Time-kill assay showed that the extracts had the strong bactericidal and fungicidal properties against planktonic cultures. The study was aimed to highlight the antibiofilm potential of *S. platensis* extracts. All the extracts enabled to inhibit biofilm formation by over 80% at doses of 1xMIC. **Conclusions.** These study results proved that the tested cyanobacterium might be a good source for producing promising antimicrobial agents against resistant bacteria. Further comprehensive research is necessary on analysis of bioactive compounds with respect to antimicrobial activity that would assist in various applications of *S. platensis* within medical practice.

**Keywords:** *Spirulina platensis*, extracts, antimicrobial activity, antibiofilm activity

### Introduction

Infectious diseases are a significant burden on public health and economic stability of societies all over the world [1].

Antimicrobial resistance has been cited as the most significant threat to the global health and global economy in recent years [2]. Bacterial antibiotic resistance is a global public health crisis, thus in 2017, the World Health Organization (WHO) created a list of microorganisms, which were prioritised for the development of alternative antimicrobials [3].

Currently, emergency action plans are required, including global community support, in developing new antimicrobial drugs [4]. Different strains of cyanobacteria and algae are

known to produce intracellular and extracellular metabolites with diverse biological activities such as antibacterial and antifungal activities [5].

*Spirulina platensis* is a planktonic filamentous cyanobacterium, showing antimicrobial activity against many pathogens (bacteria and fungi). *Spirulina* has received much attention from researchers due to its therapeutic importance in terms of bioactive compounds that act as potential antimicrobial agents [6,7].

Bacterial biofilm is currently a public health issue, affecting the population worldwide regardless of their level of development.

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The concept of biofilms is defined as new paradigm in microbiology: 99% of the bacteria on Earth live in complex bacterial consortia, being attached to a variety of surfaces, thus suggesting new research perspectives. The bacterial biofilm formation has been widely studied over the recent years. Most current studies underline the importance of biofilms in both the persistence and resistance to antimicrobials and the defense mechanisms of the human body, thus arising concerns among the clinicians [8,9].

A series of studies revealed scientific evidence on the significance of biofilms in the occurrence and development of infectious diseases. The relevant data on their ecology are crucial in providing strategies for prevention of biofilm formation and / or in treatment of biofilm-related infections.

The bacterial biofilm, involved in the occurrence of persistent infections and the evolution of antimicrobial-resistant microorganisms is particularly relevant issue nowadays. Microbial biofilms have been reported to display up to a 1000-fold more increased resistance to antimicrobials than planktonic cells, however the resistance mechanism is still unclear [9,10].

Nevertheless, a solution to this problem was provided by a series of studies, performed on the antibacterial and antibiofilm properties of several types of biological extracts. Research studies on biologically active secondary metabolites and their potential applications are far from being considered new.

The benefits of natural products lie upon their development and improvement over millions of years of evolutionary pressure, thus becoming biologically strong now. The naturally obtained substances exhibit a much higher and faster biodegradation potential, compared to artificial ones, as well as being more easily accepted on the market due to their eco-friendly aspects.

These assumptions are based on microbial diversity and their ability to adapt, therefore being able to degrade most of the natural substances. Hundreds of extracts from various plant species have been studied so far, aimed to observe their effect on microorganisms, however a relatively few samples have been sufficiently active or non-toxic to the human body. Natural products have played an important role in strengthening health and preventing infectious diseases throughout the humankind history.

Natural products are remarkable in many regards, particularly for their chemical structural diversity and biological activity, compared to substances, which were synthetically produced by the pharmaceutical industry and that rarely display a strong and biologically diverse activity [11].

However, further studies are needed on the action mechanisms and toxicity of these compounds. Therefore, research studies should be oriented towards the promising results of metabolites, synthesized by natural organisms such as cyanobacteria and microalgae, which are known for their complex and highly diverse range of metabolites, showing a variety of important biological functions.

**The purpose** of this study was to evaluate the antibacterial and antibiofilm activity of *Spirulina platensis* extracts against different bacterial species.

## Materials and methods

### ES, ES-1 and ES-2 Spirulina extracts

The biologically active complex *ES*, *ES-1* and *ES-2* extracts were obtained via a biotechnological process from the cyanobacterial strain of *Spirulina platensis* CNMN CB-02, stored within the National Collection of Non-Pathogenic Microorganisms, at the Institute of Microbiology and Biotechnology.

*Spirulina* biomass was obtained from a cyanobacterial growth culture and via a controlled synthesis of its biologically active compounds. Biologically active complexes (free amino acids and oligopeptides, proteins, sulfated polysaccharides, phospholipids) were extracted from the spirulina biomass, which were successively fractionated and purified by using innocuous solvents and techniques. Relevant formulas were developed and standardized for complex compositions of extracts, based on biologically active complexes derived from spirulina biomass. All extracts were natural ones (did not contain herbicides, toxins or preservatives).

*ES spirulina extract* is an amino acid/oligopeptide complex derived from spirulina, which contains non-essential (glycine, alanine, serine, cysteine, tyrosine, aspartic acid, glutamic acid, and proline) and essential amino acids (arginine, phenylalanine, histidine, isoleucine, leucine, lysine, threonine, tryptophan, valine, being in their free state or combined in oligopeptides (up to 10kDa), as well as biologically functionalized macro- and microelements. *In vitro* tests were used *ES aqua-alcoholic solution*, 10mg/mL (45% of ethanol).

*ES-1 spirulina extract* is a synergistic combination of amino acid/oligopeptide complex, sulfated polysaccharides, proteins, biologically functionalized macro - and microelements derived from spirulina. *In vitro* tests were used *ES-1 aqua-alcoholic solution*, 20mg/mL, (40% of ethanol).

*ES-2 spirulina extract* is a synergistic combination of amino acid / oligopeptide complex, phospholipids, sulfated polysaccharides, proteins, and biologically functionalized macro- and microelements derived from spirulina. *In vitro* tests were used *ES-2 aqua-alcoholic solution*, 20mg/mL (40% of ethanol).

### Microbial strains

Microbial strains of *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* ATCC 11778, *Enterococcus faecium* ATCC 700221, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Candida albicans* ATCC 10231, *Candida krusei* ATCC 6258 and *Cryptococcus neoformans* CECT 1043 were used in the study. The microbial strains were cultured on appropriate nutrient media at the optimum growth temperature. Overnight culture of the bacterial strains was used for further investigations.

### Antimicrobial activity

Serial culture dilutions were used to determine the antibacterial activity of plant, which allowed the assessment of the minimum inhibitory concentration (MIC) and the minimum bactericidal/fungicidal concentration (MBC/MFC). MIC and MBC/MFC were determined via a discontinuous gradient of extract concentrations tested in Muller-Hinton broth, followed by addition of 100µL of bacterial suspension according to the 0.5 McFarland turbidity standard. The tubes

were incubated at 35–37°C for 18–24 hours, and then the MIC value was determined by a macroscopic analysis of the tubes, depending on the presence or absence of bacterial growth. MBC/MFC was determined by Muller-Hinton agar dilution replicates. The MBC/MFC value displayed the lowest concentration of extract that reduced the number of microbial colonies up to 99.9% [12,13].

The negative control sample was the Muller-Hinton broth with studied extracts, and the positive control sample was the Muller-Hinton broth inoculated with studied microorganisms. All experiments were repeated three times.

### Time-Kill Assay

Time-Kill Assay allows measuring the changes in the population of aerobic microorganisms over a definite time in the presence of antimicrobial agents. Time-kill assay was carried out on *S. aureus*, *E. coli* and *C. albicans* strains, based on the aforementioned method with minor modifications [14]. A saline suspension was prepared from an 18–24 hour microbial culture, by finally obtaining an inoculum of  $1 \times 10^6$  CFU/mL. The obtained suspension was added equally into four tubes: I tube (culture control sample) - Mueller Hinton broth; II tube - Mueller Hinton broth + 0.5xMIC extract; III tube - Mueller Hinton broth + 1xMIC extract; IV tube - Mueller Hinton broth + 2xMIC extract. The tubes were incubated at 35°C for 24 hours. 100 µL from each tube was replicated on the plate medium at specific intervals. Afterwards, the plates were incubated at 37°C for 24 hours and the CFU/plate was measured, followed by CFU/mL (the mean number of colonies multiplied by dilution). The tests were performed in triplicate (three independent experiments).

Time-Kill curves were graphically represented by  $\log_{10}$  CFU mL<sup>-1</sup> versus the 24-hour time period. Bactericidal activity (99.9% of killing) was defined as a  $\geq 3 - \log_{10}$  CFU mL<sup>-1</sup> reduction in the number of colonies from the initial inoculum.

### Biofilm formation inhibition

Bacterial biofilm inhibition was assessed on seven reference strains including *S. aureus* ATCC 25923, *B. cereus* ATCC 11778, *E. faecalis* ATCC 29212, *P. aeruginosa* ATCC 9023, *E. coli* ATCC 25922, *C. albicans* ATCC 90028 and *C. neoformans* CECT 1043, being determined via the modified microtiter method [15]. The tested cultures were grown in trypticase soy broth and adjusted to the 0.5 McFarland turbidity standard. 200 µL of bacterial suspension was applied into the wells of a 96-well plate, then 100 µL of extract with final concentrations of 0.5xMIC, 1xMIC and 2xMIC was added. Only 200 µL trypticase soy broth was added into the wells of the negative control sample. The tests were performed in triplicate.

The plates were covered and incubated aerobically for 24 hours (fungi - 48 hours) at 37°C. Afterwards, the content of each well was removed and washed three times with 250 µl phosphate buffer to remove non-adherent cells. The plates were air dried and then stained with 200 µl of 0.1% purple crystal for 30 min. The dye was then removed and the excess stain was rinsed with water. The plates were air dried at room temperature and the dye, which was bound to the adherent cells, was re-solubilized with 160 µL of 33% glacial acetic acid. The results were read using an ELISA plate reader,

whereas the optical density (OD) of each well was measured at a wavelength of 570 nm (OD<sub>570 nm</sub>).

To determine the extract capacity of preventing biofilm formation, the percentage of its inhibition was calculated using the following formula:

$$\% \text{ of biofilm inhibition} = \frac{[(OD(\text{control}) - OD(\text{test})/OD(\text{control}))] \times 100}{100}$$

where OD (control) - absorption of bacterial growth without extract; OD (test) - absorption of bacterial growth in the presence of extract.

### Statistical analysis

Statistical analysis involved a one-way analysis of variance (ANOVA). A value of P less than 0.05 was considered statistically significant.

### Ethical Issues

The study was conducted and approved by the Ethics Committee no. 65/12.04.2017 and no. 67/12.05.2017 of Nicolae Testemitanu State University of Medicine and Pharmacy of the Republic of Moldova.

### Results and discussions

The infectious diseases caused by multidrug resistant pathogens are extremely challenging the healthcare systems worldwide. These disease-causing pathogens are characterized by an increased antimicrobial resistance and a marked ability to suppress the host immune response [16], as well as a high level of acquisition and dissemination of multiple resistance via horizontal gene transfer. In 80% of cases, microbial biofilms are responsible for the total number of infections, including endocarditis, periodontitis, osteomyelitis, chronic wounds, etc. [17,18]. Biofilm-related diseases are even more worrying since there are current evidence-based studies on a higher incidence rate of biofilm-mediated infections, especially due to an increased use of implantable medical devices, catheters [19].

Cyanobacteria are a group of photosynthetic microorganisms, which provide promising avenues for obtaining new compounds with high biological activity [20]. Secondary metabolites produced by cyanobacteria exhibit a wide range of biological properties, including antimicrobial activity [21]. Among cyanobacterial species, *Spirulina platensis* is the most widely studied strain by modern biotechnology worldwide, since its compounds show a high biological value, which might be used in nutraceuticals and cosmetics [22].

The development and implementation of new antimicrobial remedies, exhibiting minimal side effects on the host organism is still a very current and promising issue, particularly when identifying natural compounds with antimicrobial properties.

The antimicrobial effect of the biologically active complex extracts derived from the cyanobacterium *Spirulina platensis* were determined in the first stage of our study viz. *ES*, *ES-1* and *ES-2*.

The recent studies on spirulina extracts have reported that the extraction solvents, as methanol and ethanol, show a wide range of antibacterial and antifungal activity [23].

All the *S. platensis* extracts exhibited a promising antimicrobial activity on both gram-positive and gram-negative microorganisms used in this present research. These study findings were similar to data obtained by a number of researchers [22, 24]. In this study, *ES* extract showed a higher antibacterial and antifungal activity compared to *ES-1* and *ES-2* extracts. The highest *ES* extract activity was recorded on *B. cereus* strains (MIC 0.156 mg/mL) and *C. neoformans* (MIC 0.3125 mg/mL). The antimicrobial activity of *ES-1* and *ES-2* extracts did not show any significant differences on the tested microorganisms. The study registered a higher activity on *B. cereus* strains (*ES-1*: MIC 2.5 mg/mL; *ES-2*: MIC 1.25 mg/mL), *P. aeruginosa* (MIC 1.25 mg/mL), *C. albicans* (MIC 1.25 mg/mL) and *C. neoformans* (MIC 0.625 mg/mL). *ES* extract (containing the amino acid/oligopeptide complex derived from spirulina) was the most active among those three extracts, undergoing antimicrobial screening, thus further investigations are recommended for *in vivo* infection models testing [23, 25].

Similar results were obtained by Souza et al., WHO confirmed the antifungal properties of the methanolic *S. platensis* extract on *A. flavus* species, and the ethanolic *S. platensis* extract on the *A. niger* and *C. albicans* species [26, 27].

The methanolic *S. platensis* extract showed an antibacterial activity on *S. aureus*, *E. coli*, and *P. aeruginosa* bacterial strains [23], whereas the ethanolic *S. platensis* extract exhibited somewhat higher antibacterial activity on the following bacterial strains as, *S. thyphi*, *S. flexneri*, *E. coli*, *S. aureus*, *P. aeruginosa*, and *Klebsiella* spp. [25].

**Time-killing kinetics.** The studies of time-killing kinetics of *ES*, *ES-1* and *ES-2* extracts on *S. aureus*, *E. coli* and *C. albicans* strains have proved a 24-hour viability of control sample growth in Mueller-Hinton broth without extracts. Moreover, the studied bacterial strains, treated with *ES*, *ES-1* and *ES-2* extracts at concentrations of 0.5x, 1x and 2xMICs showed a considerably reduced growth rate, confirming that all extracts display bactericidal and fungicidal properties by killing > 90% of microbial cells.

The bactericidal and fungicidal effect of the extracts was faster (1 hour for *S. aureus* and *E. coli* strains and 2-4 hours for *C. albicans*) at the concentration of 2xMIC for all tested extracts compared to other MICs (0.5xMIC and 1xMIC). *ES* proved a higher activity on fungi of genus *Candida*, showing a 2-hour fungicidal effect at a concentration of 1xMIC and an 8-hour effect at a concentration of 0.5 MIC.

*ES-1* extract showed the highest activity on *E. coli* strains, with a 3-hour bactericidal effect at 1xMIC compared to *ES* and *ES-2*, which had a 10-hour bactericidal effect. *S. aureus* strains were destroyed by all preparations over 10 hours, at a concentration of 1xMIC.

The bacterial biofilms involved in the occurrence of persistent infections and the evolution of multidrug-resistant antimicrobial microorganisms have generated a series of studies regarding the antibiofilm mechanisms of action, as well as on the development of new effective and low toxicity drugs.

Most research studies highlight the importance of antimicrobial resistance of biofilm-producing microorganisms, compared to planktonic ones, as well as the immunosuppressive effects of the host organism due to a chronic infection

[10, 28]. Mature biofilms are much more difficult to eradicate than prevent the early stages of bacterial attachment in biofilm formation [29].

A previous study was conducted on the methanolic *S. platensis* extract, which was tested on clinically selected important bacterial strains. The studied extract showed inhibitory effects on the biofilm formation: 69% and 72% (*P. aeruginosa* MTCC1934 and 2453), 62% (*E. coli* MTCC739), 84% and 61% (*S. aureus* MTCC96 and 2940), 89% (*C. violaceum* ATCC 12742), 49% (*S. marcescens*), 74% (*A. hydrophila* MTCC1739), 88% (*V. alginolyticus* ATCC17749) and 90% (*V. parahaemolyticus* ATCC17802) [30].

Other researchers confirmed the anti-biofilm activity of marine bacteria extracts, which showed 85% maximum potential against Gram-positive and Gram-negative bacteria at a concentration of 50 µg/mL [31].

Despite the great number of studies on the multiple potential effects of *S. platensis*, its effect on microbial biofilms has not fully been elucidated, becoming a matter of great concern for modern medicine. The current study enabled to highlight the anti-biofilm potential of *S. platensis* extracts against both gram-positive and gram-negative microorganisms. The three extracts of *S. platensis* exhibited a variety of effects on biofilm growth.

All the extracts were able to inhibit the biofilm formation with more than 80% at doses of 1xMIC. The studied extracts inhibited the biofilm-producing capacity of the strains in 100% of cases at doses of 2xMIC, showing a statistically significant difference between these two concentrations ( $P < 0.001$ ).

The obtained results are relevant to a number of previous studies regarding the anti-biofilm activity of *S. platensis* extracts, both on gram-positive and gram-negative microorganisms [30].

## Conclusions

*Spirulina platensis* is a valuable, non-conventional, and eco-friendly source, as well as one of the most exploited cyanobacteria for obtaining and exploiting the biologically active compounds with high therapeutic potential.

The research studies regarding the action of polycomponent *Spirulina platensis* extracts on the bacterial strains have proved their *in vitro* antimicrobial and anti-biofilm properties, thus being recommended for *in vivo* infection models testing.

The study has provided experimental-based evidence of bacterial and fungal biofilm inhibition by the following *Spirulina platensis* extracts: *ES*, *ES-1* and *ES-2*, thus suggesting their efficacy in controlling chronic and persistent infections caused by these microorganisms.

The studied *Spirulina platensis* extracts represent complexes of natural compounds, which have generated significant and promising medical interest on their use for developing natural antibiotics against multidrug-resistant bacteria and infectious diseases.

**Conflicts of interests.** Authors declare the absence of any conflicts of interests and their own financial interest that might be construed to influence the results or interpretation of their manuscript.

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## ОЦЕНКА *IN VITRO* АНТИМИКРОБНОЙ И ИНГИБИРУЮЩЕЙ БИОПЛЕНКУ АКТИВНОСТИ ЭКСТРАКТОВ *SPIRULINA PLATENSIS*

**Резюме. Актуальность.** За последние десятилетия частота микробных инфекций увеличилась из-за увеличения количества людей с ослабленным иммунитетом, а также из-за развития устойчивости у микроорганизмов, вовлеченных в их этиологию. Повышение устойчивости микробных штаммов к антибиотикам, наряду с образованием биопленок, являются серьезными проблемами в борьбе с инфекциями, вызываемыми этими организмами. В настоящее время из природных источников получено внушительное количество противомикробных препаратов. Некоторые виды цианобактерий, такие как *Spirulina platensis*, могут продуцировать большое количество антимикробных веществ и считаются подходящими организмами для использования в качестве противомикробных агентов против патогенных бактерий и грибов. **Целью** этого исследования было определение антимикробной и антибиопленочной активности экстрактов *Spirulina platensis*. **Материалы и методы.** Антибактериальную активность оценивали с помощью определения минимальной ингибирующей концентрации (МИК), минимальной бактерицидной/ фунгицидной концентрации (МБК/МФК), а также time-kill kinetics тестов. Образование биопленок

определяли методом микротитрационного планшета. Статистический анализ включал односторонний дисперсионный анализ. **Результаты.** Настоящее исследование доказало антибактериальную, противогрибковую и антибиопленочную активность экстрактов *Spirulina platensis* (ES, ES-1 и ES-2) *in vitro*. Time-kill анализ показал, что экстракты обладают сильными бактерицидными и фунгицидными свойствами против планктонных культур. Исследование было направлено на выявление антибиопленочного потенциала экстрактов *S. platensis*. Все экстракты позволили подавить образование биопленок более чем на 80 % в дозах 1хМИК. **Выводы.** Результаты этих исследований доказали, что испытанные цианобактерии могут быть хорошим источником для получения многообещающих антимикробных средств против устойчивых бактерий. Необходимы дальнейшие всесторонние исследования этих биологически активных соединений в отношении их противомикробной активности, которые могут помочь определить различные возможности применения *S. platensis* в медицинской практике.

**Ключевые слова:** *Spirulina platensis*; экстракты; антимикробная активность; антибиопленочная активность

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## ОЦІНКА *IN VITRO* АНТИМІКРОБНОЇ ТА ІНГІБУЮЧОЇ БІОПЛІВКУ АКТИВНОСТІ ЕКСТРАКТІВ *SPIRULINA PLATENSIS*

**Резюме. Актуальність.** За останні десятиліття частота мікробних інфекцій збільшилася через збільшення кількості людей з ослабленим імунітетом, а також через розвиток стійкості в мікроорганізмів, залучених в їх етіологію. Підвищення стійкості мікробних штамів до антибіотиків, поряд з утворенням біоплівок, є серйозними проблемами у боротьбі з інфекціями, спричиненими цими організмами. У даний час з природних джерел отримано значну кількість протимікробних препаратів. Деякі види ціанобактерій, такі як *Spirulina platensis*, можуть продукувати велику кількість антимікробних речовин і вважаються придатними організмами для використання в якості протимікробних агентів проти патогенних бактерій і грибів. **Метою** цього дослідження було визначення антимікробної та антибіоплівкової активності екстрактів *Spirulina platensis*. **Матеріали та методи.** Антибактеріальну активність оцінювали за допомогою визначення мінімальної інгібуючої концентрації (МІК), мінімальної бактерицидно/фунгіцидної концентрації (МБК/МФК), а також time-kill kinetics тестів. Утворення біоплівок ви-

значали методом микротитраційного планшета. Статистичний аналіз включав односторонній дисперсійний аналіз. **Результати.** Це дослідження довело антибактеріальну, протигрибкову і антибіоплівкову активність екстрактів *Spirulina platensis* (ES, ES-1 і ES-2) *in vitro*. Time-kill аналіз показав, що екстракти мають сильні бактерицидні та фунгіцидні властивості проти планктонних культур. Дослідження було спрямоване на виявлення антибіоплівкового потенціалу екстрактів *S. platensis*. Усі екстракти дозволили придушити утворення біоплівки більш ніж на 80 % у дозах 1хМІК. **Висновки.** Результати цих досліджень довели, що випробувані ціанобактерії можуть бути хорошим джерелом для отримання багатообіцяючих антимікробних засобів проти стійких бактерій. Необхідні подальші всебічні дослідження цих біологічно активних сполук щодо їх протимікробної активності, які можуть допомогти визначити різні можливості застосування *S. platensis* у медичній практиці. **Ключові слова:** *Spirulina platensis*; екстракти; антимікробна активність; антибіоплівкова активність