Short Communication

Natural Product Communications
Volume 20(1): 1–6

DOI: 10.1177/1934578X251314702
journals.sagepub.com/home/npx
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In Vitro Antibacterial and Antifungal Activity of an *Arthrospira platensis* (Syn.: *Spirulina platensis*) Extract

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Abstract

Objective: To assess the antibacterial and antifungal activity *in vitro* of a patented Spirulina extract on sensitive and multidrug-resistant bacteria, yeasts, and filamentous fungi commonly causing skin infections. **Methods:** The antimicrobial activity was tested using the drop dilution assay on the Gram-positive bacteria *Staphylococcus aureus* (wildtype), a methicillin resistant *S. aureus, S. epidermidis*, *S. haemolyticus*, *Streptococcus pyogenes*, the Gram-negative multidrug resistant *Pseudomonas aeruginosa*, the yeasts *Candida albicans* and *C. tropicalis*, and the dermatophytes *Trichophyton rubrum* and *Microsporum gypseum*. **Results:** A concentration of 4 g of Spirulina extract per 100 mL of Mueller Hinton agar completely inhibited the growth of all tested bacteria and yeasts, and suppressed dermatophytes growth by 5 $\log_{10}$ units. **Conclusion:** The Spirulina extract tested could be used as an effective natural, broad-spectrum antimicrobial agent, with potential applications in the therapy of bacterial and fungal infectious diseases.

Keywords

antibiotic susceptibility testing, drop test, methicillin resistant *Staphylococcus aureus*, MRSA, multidrug resistance, skin infection, Spirulina extract

Received: September 11th, 2024; Accepted: January 6th, 2025.

Introduction

Cyanobacteria, formerly also incorrectly called "blue-green algae", are important producers of secondary metabolites that have been shown to act *in vitro* against bacteria,[1] fungi,[2] viruses,[3] as well as on protozoa and molluscs.[1]

Arthrospira platensis (synonym: *Spirulina platensis*), also known under the commercial name Spirulina, is widely used as a dietary supplement, for which the United States Pharmacopeia has issued a Class A safety rating, thus allowing the admission of quality monographs as a dietary supplement.[4] Medically, Spirulina's properties have been described as hypolipidemic, antioxidant, and anti-inflammatory.[5] So far, however, clinical evidence for any beneficial activity of Spirulina supplements, be they powders or extracts, is scant and mostly limited to their cholesterol-lowering potential.[6]

Several *in vitro* and *in vivo* studies suggest a strong antibacterial, antifungal, and antiviral activity of Spirulina extracts,[6-8] but active pharmaceutical ingredients responsible for this antibacterial and antifungal activity have not yet been identified. Spirulina produces several bioactive compounds, including phenolics, fatty acids, glycolipids, and phycocyanins.[9] These compounds may disrupt bacterial cell walls, inhibit bacterial growth, or interfere with the metabolism of microorganisms. Overall, however, Spirulina extracts have not yet been fully characterized with regards to their active ingredients. While several publications dealt with some physicochemical properties,[10-15] no studies have been published suggesting the impact of selected components on the medical properties of Spirulina, nor have convincing *in vitro* or *in vivo* experiments been carried out to elucidate the antibiotic activity of the whole extracts or any of its components.

Previous work has reported the presence of phenolic compounds with potent antioxidant activity in Spirulina extracts[13] that could be expected to be at least partly responsible for the antibiotic activity observed.[11] The highly polar compounds contained in the extracts,[16] notably calcium spirulan (CaSP),

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a sulfated polysaccharide chelating calcium,[17] were described to exert a marked anti-herpes and anti-human immunodeficiency virus (HIV) activity.[18]

A patented Spirulina extract has been shown in an observational study in humans[19] to be superior to topical acyclovir cream in preventing cold sore recurrence. Data on its antifungal and antibacterial activity, however, are scant. Only unpublished results from *in vitro* experiments[20] and summary information presented in a US patent[21] have suggested that this extract could exert antibacterial and antifungal effects. The antibiotic activity observed is likely to be the result of a synergistic action of its components, a feature described for several herbal medicinal products.[22,23] Therefore, a necessary step in the development of a Spirulina extract to be used in the therapy of infectious diseases consists of assessing the overall antibiotic activity of the extract on sensitive and multidrug resistant bacteria before embarking in more detailed analyses and tests of individual components. This study was thus designed to confirm *in vitro* the antibacterial and antifungal activity of this patented extract using validated test conditions and appropriate controls on a range of sensitive and multidrug resistant bacteria, yeasts, and dermatophytes commonly present on the skin and frequently causing infections, thrush and dermatophytoses.

Results

A concentration of 1 g/100 mL Spirulina extract exerted mostly a complete growth inhibition, the only exceptions being *S. epidermidis* (reduction from 10^6 to 10 CFU) and *Str. pyogenes*, for which growth was inhibited by only 1 $\log_{10}$ unit from 10^6 to 10^5 CFU (Figure 1). However, at a concentration of 4 g/100 mL, none of the bacterial strains tested were able to grow (Figure 1).

Candida albicans growth was already inhibited in the presence of 1 g/100 mL extract, but only a concentration of 4 g/mL extract completely suppressed *C. tropicalis* growth (Figure 2). While at 1 g/100 mL the extract was barely active against *M. gypseum* (reduction by 3 $\log_{10}$ units), *Trichophyton rubrum* growth, at this concentration, was already reduced by 5 $\log_{10}$ units; 4 g/mL extract reduced the growth of both dermatophytes by 5 $\log_{10}$ units (Figure 2).

Discussion

The patented Spirulina extract, already at a concentration of 1 g/100 mL, exerted a strong inhibitory effect on almost all Gram-positive bacteria tested and *Candida albicans*. At a concentration of 4 g/100 mL a complete suppression of the growth of all bacterial and yeast strains tested was observed, proving that the extract is a potent antimicrobial agent against Gram-positive, Gram-negative bacteria, and yeasts. At a concentration of 1 g/100 mL the extract was active on *T. rubrum* and only marginally on *M. gypseum*, but a 4 g/100 mL concentration almost completely suppressed growth of both dermatophytes tested, by reducing growth from 10^6 to 10 CFU.

Several studies have shown that Gram-positive and Gram-negative bacteria, yeast and filamentous fungi are susceptible to purified biomolecules obtained from *A. platensis* and other microalgae.[24] The present results agree with these findings and previous work summarized in a recent review.[8]

Some authors have suggested that Spirulina extracts could be used to treat at least mild infectious diseases of the skin,[11] without providing any experimental data. There is a need to study potentially active compounds derived from cyanobacteria in validated *in vitro* models, to develop new antimicrobial agents that could be used complementarily or as alternatives to classical antibiotics for the treatment of skin infections caused by some Gram-positive *Staphylococcus* species and *Streptococcus pyogenes*,[25] as well as by some *Candida* species[26] and dermatophytes.[27-29] Novel therapeutic agents against bacterial and fungal infections are urgently required, as multidrug resistant bacteria and fungi are increasingly becoming a problem,[30] and the results of this study suggest that Spirulina's use as an antimicrobial agent could become an interesting area of research. Several active substances present in Spirulina, among them the oligosaccharide PO-1, have been shown to exert significantly positive regulatory effects on intestinal *Lactobacillus*, *Akkermansia*, *Arthromitus*, *Butyricimonas*, and *Prevotella*, and to reduce the proportion of harmful bacteria such as *Dorea* and *Clostridium*.[31] These beneficial activities are now complemented by the antimicrobial efficacy described here.

The broad-spectrum activity of the Spirulina extract tested on multidrug resistant bacteria and filamentous fungi could be valuable for the development of new treatments against resistant pathogens. So far, the extract is used only in cosmetic products, but our results suggest that a targeted pharmacological development to treat at least mild infectious skin conditions should be considered.

The results presented here are the first proof of the antibacterial and antifungal activity of a Spirulina extract carried out in a validated and reproducible assay. More *in vitro* experiments and studies in animal models are needed before this extract can be used in clinical studies, but these data will already benefit researchers working with other Spirulina extracts.

So far, the active pharmaceutical ingredients responsible for the antibiotic activity of Spirulina have not been identified. As for other products of plant or bacterial origin, it can be expected that the activity does not stem from a single ingredient, but an additive or possibly even a synergistic activity of several compounds can be expected, which would fit previous observations made with other herbal medicines (see, eg, Wagner[32]). Overall, the antimicrobial metabolites from Spirulina hold a high pharmaceutical potential. The broad antimicrobial activity observed in this study justifies further research that may lead to the development of natural products to be used against multidrug resistant pathogens.

Experimental

Spirulina extract: The extract used in this study is derived from a Spirulina powder (ILU, Bad Belzig, Germany) produced using

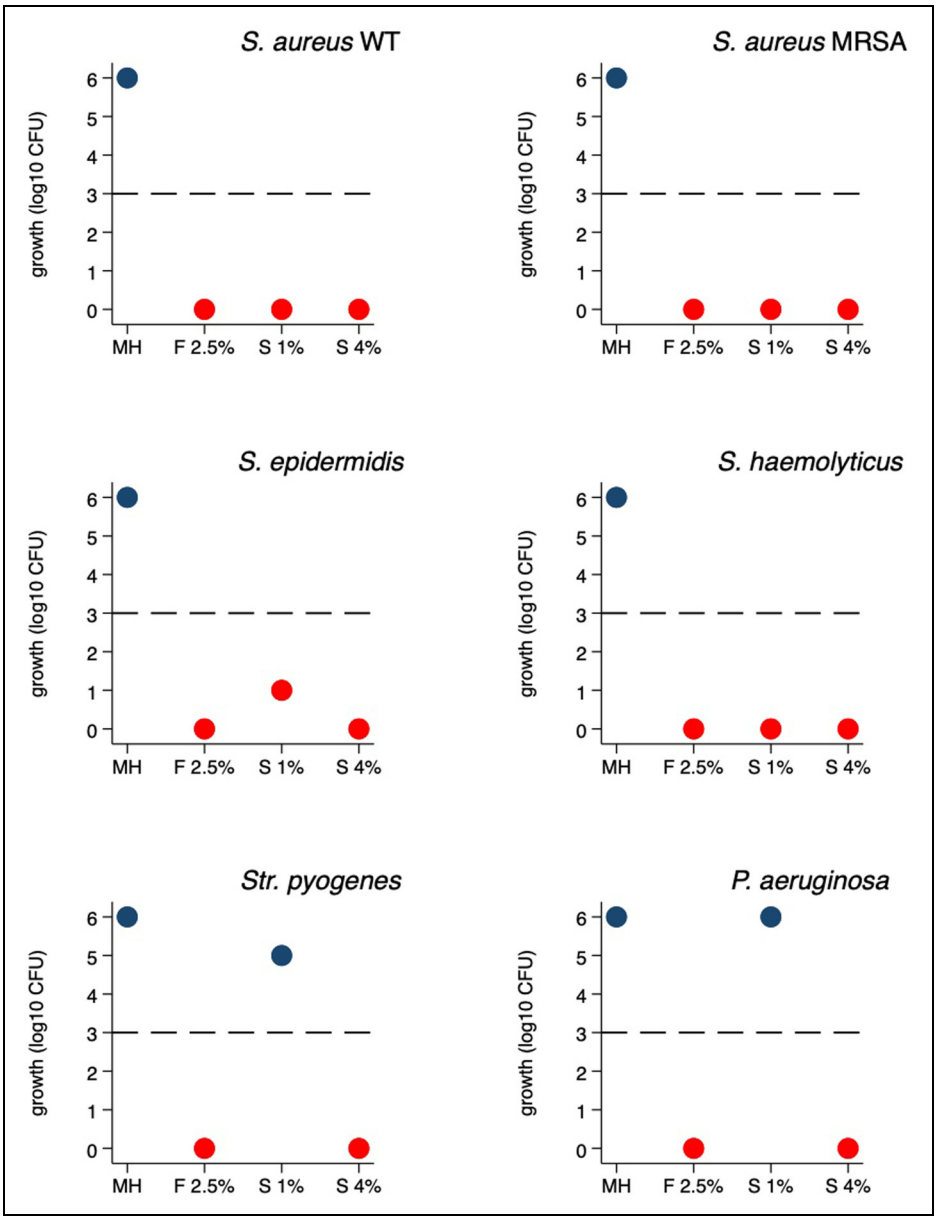

Figure 1. Antibacterial activity of the Spirulina extract on the bacteria tested. Results are the mean of 8 replicates. Red dots indicate that the strains have been inhibited in their growth, whereas blue dots denote no effect of the compound tested: 6: no inhibition (the microorganisms grew in all drops from 10-10^6 CFU/mL) and 0: maximum inhibition (no growth observed at any concentration). A biologically significant inhibition was arbitrarily defined as no or reduced growth at a concentration of 10^3 CFU/mL ($\log_{10}$ value of 3, dashed line). MH: Müller-Hinton (positive control, no growth inhibition); F 2.5%: 2.5% Fucidin ointment (negative control); S 1%: 1% Spirulina extract; S 4%: 4% Spirulina extract.

A. platensis cultures from Myanmar. The powder is sun-dried and the final product certified to be free from genetically modified organisms, artificial colorants, preservatives or additives, pesticides, algal toxins, and allergens; its organoleptic properties (color, smell, consistence) and moisture, protein, lipid and mineral contents are constantly monitored to guarantee a batch-to-batch uniformity. The extract is generated using a patented extruder method, using water as solvent followed by an ethyl acetate extraction, as described in detail by Keimes and Günther.[21] This process provides controlled stress conditions that allow on the one hand increasing the yield of (hitherto undetermined) pharmacologically active substances contained in the powder, on the other to eliminate batch-to-batch variations of the extract through a standardized manufacturing process. The extract is released according to physico-chemical and microbiological specifications, such as pH, solubility in ethanol, particle size, heavy metal content, and bacterial contamination.

Strains and antimicrobial agents: The antibacterial and antifungal activity of the extract was tested on the Gram-positive

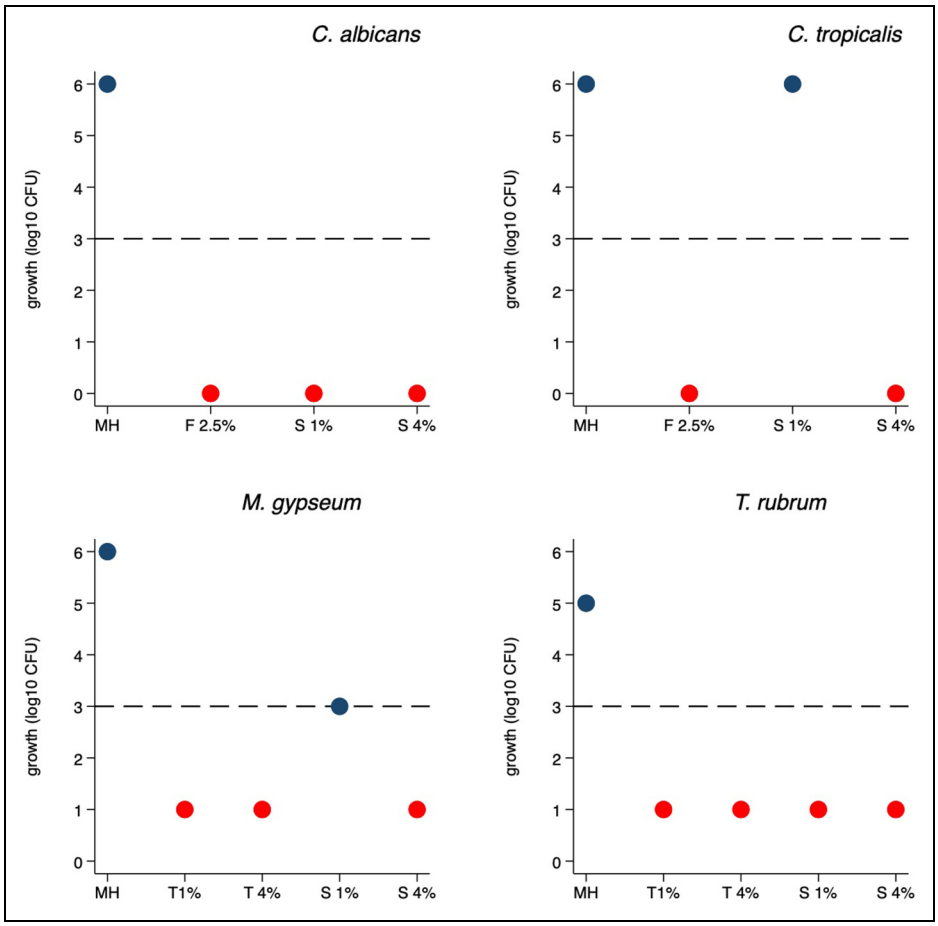

Figure 2. Antifungal activity of the Spirulina extract on the yeasts and fungi tested. Results are the mean of 8 replicates. Red dots indicate that the strains have been inhibited in their growth, whereas blue dots denote no effect of the compound tested: 6: no inhibition (the microorganisms grew in all drops from 10-10^6 CFU/mL) and 0: maximum inhibition (no growth observed at any concentration). A biologically significant inhibition was arbitrarily defined as no or reduced growth at a concentration of 10^3 CFU/mL ($\log_{10}$ value of 3, dashed line). MH: Müller-Hinton (positive control, no growth inhibition); F 2.5%: 2.5% Fucidin ointment (negative control for yeasts); S 1%: 1% Spirulina extract; S 4%: 4% Spirulina extract. T 1%: 1% Terbinafine ointment (negative control for dermatophytes); T 4%: 4% Terbinafine ointment (negative control for dermatophytes).

bacteria *Staphylococcus aureus* (wild type, ATCC 23923), a methicillin resistant *S. aureus* (MRSA) strain (ATCC 700699), *S. epidermidis* (ATCC 12228), *S. haemolyticus* (SMIC 01), and *Streptococcus pyogenes* (ATCC 19615), the Gram-negative *Pseudomonas aeruginosa* (ATCC 27853), the yeasts *Candida albicans* (ATCC 14053) and *C. tropicalis* (SMIC 02), and the filamentous fungi *Microsporum gypseum* (SUPSI 01) and *Trichophyton rubrum* (SUPSI 02). All strains were grown overnight on Mueller–Hinton agar (MHA, Oxoid) without supplementation at 37 C. Filamentous fungi were incubated for 10-15 days at 37 C and regularly checked for microconidia production.

Growth media preparation: The concentrations of the Spirulina extract in the growth medium (1 and 4 g/100 mL of MHA) were selected to reflect the posology of a marketed product (Spirularin® NF ointment and mousse information leaflet, ocean pharma GmbH). A cream containing fusidic acid (Fucidin 2% ointment, LEO Pharma A/S, Ballerup, Denmark), a topical antibiotic commonly used to treat bacterial skin infections,[28] was used at a concentration of 2.5 g/100 mL of MHA as a control for bacterial and yeast growth inhibition, as this concentration was shown to be active in preliminary experiments (data not shown). A cream containing terbinafine (10 mg/g ointment, Mepha Pharma AG, Basel, Switzerland), a topical antimycotic active against fungal infections of skin, hair and nails, was used as control for the growth inhibition of the filamentous fungi at concentrations of 1 and 4 g/100 mL of MHA.

All test substances were added to autoclaved MHA, cooled to approximately 50 C, and thoroughly mixed with the medium.

Antibiotic activity testing: A drop dilution test[33] was used to assess to determine the inhibitory activity of the extract on viable cells and spores in a liquid suspension. After incubation, the growth of colonies in each drop is visually evaluated. For bacteria and yeasts, the microorganisms were drop-seeded on MHA supplemented with different concentrations of the extract. The bacterial and yeast inoculum were prepared by culturing the microorganisms overnight and resuspending them in 0.85% NaCl. The suspensions were then adjusted to a final

density of 1.5×10^8 colony forming units (CFU) per mL, at a final optical density of 0.5 McFarland for bacterial and 1 McFarland for yeast strains as recommended in the EUCAST guidelines.[34] Serial dilutions of the bacterial and yeast cells were from 10^8 to 10^3 CFU/mL. For filamentous fungal strains, the inoculum was obtained by gently scraping the fungal surface with a sterile swab to collect microspores that were resuspended in 0.85% NaCl solution and adjusted to 1.5×10^7 spores/mL by cell counting in a Neubauer chamber. Serial dilutions of the spore suspensions were from 10^7 to 10^3 spores/mL, taking care of having almost exclusively microconidia in the suspensions. Ten µL of each dilution were spotted on MHA supplemented with the extract, the antibiotic or the antimycotic compounds.

Bacteria and yeasts were incubated at 37 °C for 2 days, and filamentous fungi at 37 °C for 7 days under standard conditions.

Measurement of inhibition and data analysis: To determine growth inhibition of the bacterial, yeast and filamentous fungi strains by the extract, the growth on the positive control (MHA: no inhibition) was used as reference (growth: 100%). All experiments were performed in quadruplicates on two different days, thus 8 replicates for each strain tested at the different growth conditions were available for evaluation. No formal confirmative statistical analysis was carried out. Inhibition is presented graphically as means of the 8 replicates (ie, the mean concentration at which no growth was observed). As these data are only semi-quantitative and could be considered categorical, we did not compute any standard deviation or the corresponding non-parametrical counterparts (percentiles). Literature data estimate that the abundance of bacterial and yeast cells on skin is $10^3 - 10^4/cm^2$, increasing to 10^6-$10^7/cm^2$ in wet areas or in case of skin infections.[35] A biologically significant inhibition was thus arbitrarily defined as no growth at a concentration of 10^3 CFU/mL (log 10 value of 3, dashed line in the graphical displays). The same approach and cutoff value was taken for the two dermatophytes, as no reliable estimates on colonization or infectivity values exist for these pathogens.

Graphical displays of the results were prepared using R version 4.3.2,[36] using the packages ggplot2[37] and ggsci.[38]

Acknowledgements

SMIC strains have been kindly supplied by the Clinical Microbiology Service (SMIC) of the cantonal hospital of Ticino, Switzerland. The Spirulina extract was kindly provided by ocean pharma GmbH, Dieselstrasse 6, D-21465 Reinbek, Germany.

Author Contributions

Conceptualization: NG, PG and CZ. Methodology: CF, EP and OP. Investigation: CF and EPs. Writing – original draft: CF, EP, OP. Writing – Review and Editing: CF, EP, OP, PG, CZ and NG. Visualization: JDM, OP. All authors gave final approval of the manuscript and are accountable for all aspects of the work.

Declaration of Conflicting Interests

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: EP and CF received a grant from Cesra GmbH & Co. KG to carry out the laboratory experiments. OP is a consultant to Cesra. CZ and NG are employees of Cesra.

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