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Bioactive Extracts of *Spirulina platensis* Inhibit *Colletotrichum orchidearum* and *Fusarium nirenbergiae*: A Green Approach to Hydroponic Lettuce Protection

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Abstract

The growing demand for food and the environmental impact of conventional agriculture have prompted the search for sustainable alternatives. Phycocyanin (PC) and total phenolic compounds (TPC) extracted from *Spirulina platensis* have shown potential for the biological control of phytopathogens. The extraction method directly influences the yield and stability of these compounds. This study aimed to establish an efficient extraction protocol for PC and TPC and to evaluate their antimicrobial efficacy in vitro against *Colletotrichum orchidearum*, *Fusarium nirenbergiae*, and *Alternaria* sp. isolated from hydroponically grown lettuce. The phytopathogens were identified based on phylogenetic analyses using sequences from the ITS, EF1-α, GAPDH, and RPB2 gene regions. This is the first report of *C. orchidearum* in hydroponic lettuce culture in Brazil, expanding its known host range. Extracts were obtained using hydroalcoholic solvents and phosphate buffer (PB), combined with ultrasound-assisted extraction (bath and probe). The extracts were tested for in vitro antifungal activity. Data were analyzed by ANOVA ($p < 0.05$), followed by Tukey's test. The combination of the PB and ultrasound probe resulted in the highest PC (95.6 mg·g^{-1} biomass) and TPC (21.9 mg GAE·g^{-1}) yields, using 10% (w/v) biomass. After UV sterilization, the extract retained its PC and TPC content. The extract inhibited *C. orchidearum* by up to 53.52% after three days and *F. nirenbergiae* by 54.17% on the first day. However, it promoted the growth of *Alternaria* sp. These findings indicate that *S. platensis* extracts are a promising alternative for the biological control of *C. orchidearum* and *F. nirenbergiae* in hydroponic systems.

Keywords: biocontrol; phytopathogens; microalgae; phycocyanin; total phenolic compounds

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1. Introduction

Intensive agricultural practices, such as the indiscriminate use of chemical agents for disease control and fertilizers, have contributed to environmental pollution and reinforced the urgent need to minimize the negative impacts on human health and the environment resulting from the widespread use of these inputs [1]. In this context, there is increasing interest in developing more sustainable alternatives such as biocontrol products and biofertilizers capable of improving plant nutrition and acting against phytopathogens [2].

Microalgae have been explored as an innovative approach with which to promote plant growth, enhance productivity, and mitigate biotic and abiotic stresses [3]. *Spirulina platensis*,

a blue-green cyanobacterium, contains a wide range of pigments, such as carotenoids, chlorophyll, and phycobiliproteins (e.g., phycocyanin), which capture approximately 50% of the light required for photosynthesis [4].

There is growing interest in the use of algal-derived compounds for plant health management [5,6]. The weekly application of aqueous extracts of *S. platensis* in hydroponically grown lettuce (*Lactuca sativa* L.) led to earlier plant maturity compared to the control group, highlighting its potential as a biostimulant in horticulture [2]. Furthermore, the inhibitory activity of *S. platensis* against *Fusarium oxysporum* was demonstrated by a 45% reduction in disease incidence in pepper plants following foliar applications three times per week, using a suspension containing 10^7 spores·mL^{-1} [7].

Lettuce, widely cultivated in hydroponic systems, is susceptible to several fungal diseases that compromise crop productivity and quality [8]. Despite the reduced contact with soil, hydroponic cultivation does not ensure plant health or microbiological safety [9]. Pathogens spread rapidly in these systems due to the recirculation of the nutrient solution, affecting all plants within the setup [10]. Among the primary pathogens associated with hydroponically grown lettuce, the *F. oxysporum* species complex (FOSC) is particularly concerning, as it causes vascular wilt, leading to chlorosis, collapse of the aerial parts, and ultimately plant death [11,12].

Colletotrichum spp. also infects various crops, including lettuce, and is responsible for anthracnose, which can result in yield losses exceeding 50% in agricultural production [13,14]. Among the *Colletotrichum* species known to infect lettuce, this study is the first to report on the isolation of *C. orchidearum* from hydroponically grown lettuce in Brazil. Originally described in 2018 and mostly associated with ornamental plants [15], this finding expands on the known host range of this pathogen and highlights a new challenge for disease management in hydroponic systems. In addition, *Alternaria* spp. may be associated with lettuce seeds and, in mature plants, can cause Alternaria leaf spot—characterized by necrotic lesions on leaves, petioles, flowers, and inflorescences—which may lead to plant lodging and death [16,17].

Phycocyanin (PC) is a natural blue pigment with applications in food, nutraceutical, and biotechnological industries [18–20]. The efficient extraction of bioactive compounds is a key factor for the utilization of biomolecules with industrial and biotechnological value. Among the most relevant compounds, total phenolic compounds (TPC) and PC present important functional properties such as antioxidant activity and antimicrobial potential. The extraction method directly influences the yield and stability of these compounds. Aqueous solvents, such as phosphate buffer, are commonly used to preserve the integrity of water-soluble proteins and pigments, while hydroalcoholic mixtures are more effective for extracting TPCs of different polarities [4,21].

The extraction of bioactive compounds from microalgal biomass involves mixing dried biomass with solvents such as ethanol, dimethyl sulfoxide, or phosphate buffer solutions [21–23]. To enhance the release of intracellular compounds, technologies, such as ultrasonic bath and/or ultrasound probe application, have proven effective [24,25]. However, few studies have applied these technologies to the extraction of bioactive compounds with potential use in agriculture, particularly in plant disease management.

Therefore, this study aimed to develop an extraction method for PC and TPC from *S. platensis* biomass and to evaluate their in vitro application for the biological control of *C. orchidearum*, *F. nirenbergiae*, and *Alternaria* sp. isolated from hydroponically grown lettuce, contributing to the development of sustainable alternatives for plant disease management.

2. Materials and Methods

2.1. Materials and Chemicals

All solvents, reagents, and chemicals used in this study were of analytical grade. The *S. platensis* biomass used in this study was commercially obtained from Mocó Agropecuária (Fazenda Tamanduá, Santa Teresinha, PB, Brazil), batch number 91, with a declared purity of 100%. The phosphate buffer was prepared using monobasic sodium phosphate (NaH_2PO_4, PA grade, Neon, Diadema, SP, Brazil) and dibasic sodium phosphate (Na_2HPO_4, PA grade, Neon, Diadema, SP, Brazil). Absolute ethanol (99.8% PA) was obtained from Dinâmica (Indaiatuba, SP, Brazil) and used in the preparation of hydroalcoholic extracts. Streptomycin sulfate (PA grade, Dinâmica, Indaiatuba, SP, Brazil) was added to the culture media to inhibit bacterial growth. Bovine serum albumin (BSA, $\geq$98%, Sigma-Aldrich, St. Louis, MO, USA) was used to prepare the standard curve for protein quantification. Carbohydrate content was determined using anhydrous glucose (PA grade, Neon, Diadema, SP, Brazil) as the standard.

For total phenolic compound (TPC) analysis, hexane (PA grade, Neon, Diadema, SP, Brazil) was used for extract purification, followed by treatment with barium hydroxide (PA grade, Neon, Diadema, SP, Brazil) and zinc sulfate heptahydrate (PA grade, Neon, Diadema, SP, Brazil). The Folin–Ciocalteu reagent, and gallic acid standard (GAE) (Sigma-Aldrich, St. Louis, MO, USA) were used for TPC quantification.

The culture medium used for fungal growth was potato dextrose agar (PDA, Kasvi, Pinhais, PR, Brazil). For DNA extraction, the Wizard® Genomic DNA Purification Kit and the ReliaPrep™ DNA Clean-Up and Concentration System were obtained from Promega (Madison, WI, USA). PCR products were purified using the ReliaPrep™ DNA Clean-Up and Concentration System. Sequencing was performed in both directions using the same PCR primers by ATCGene molecular analysis (Campinas, SP, Brazil) on an AB-3500 automatic sequencer (Applied Biosystems, Waltham, MA, USA). A 1 kb molecular weight marker was purchased from Qiagen (Germantown, MD, USA), and blue-green loading dye was obtained from LGC (Cotia, SP, Brazil).

Spectrophotometric measurements were performed using a UV-1600 spectrophotometer (Pro-Tools, São Paulo, SP, Brazil), and DNA concentrations were determined with a NanoDrop 2000 (Thermo Scientific, Waltham, MA, USA). Electrophoresis gels were visualized using the Safe Imager Blue Light Transilluminator (Invitrogen, USA). The ultrasonic probe system used for sonication was the DES500 (Unique, Linhares, ES, Brazil); and the ultrasonic bath was the P60H (Elma Ultrasonic, Singen, Germany). Homogenization was performed using an Ultra-Turrax T25 (IKA, Campinas, SP, Brazil); centrifugation was conducted using a CIENTEC CT-5000 centrifuge (Porto Alegre, RS, Brazil). Ethanol removal from the extracts was carried out using a rotary evaporator (TE-211, TECNAL, Piracicaba, SP, Brazil).

2.2. Isolation and Identification of Phytopathogens

Phytopathogens were isolated from lettuce plants grown in hydroponic systems that showed visible symptoms of root diseases. A large number of diseased lettuce roots were collected for the isolation procedure. The diseased roots were washed with detergent and water to remove debris and/or substrate; symptomatic tissues were cut into 1 cm sections [26].

The fragments were disinfected with a 1% (v/v) sodium hypochlorite solution, prepared by diluting commercial bleach containing 2% (w/v) sodium hypochlorite with sterile distilled water. The fragments were immersed in this solution for 1 min, rinsed twice with sterile distilled water, and dried on paper towels in a laminar flow cabinet for 5 min. Approximately five fragments were transferred to each of ten Petri dishes (90 mm $\times$ 15 mm)

containing potato dextrose agar (PDA) supplemented with streptomycin sulfate (0.20 $g \cdot L^{-1}$) to prevent bacterial growth. The plates were incubated at 25 °C under a 12 h photoperiod and monitored for hyphal growth. Hyphal tips were subcultured onto fresh PDA plates supplemented with streptomycin sulfate until pure cultures were obtained [26].

For identification, a 5 mm diameter mycelial disc from each pure culture was transferred to a Petri dish containing PDA medium. The plates were incubated for 5 days at 25 °C under a 12 h photoperiod. The microorganisms were sent for identification to Agronômica Laboratory—Plant Health Diagnostic and Consulting Laboratory, located in Porto Alegre, Rio Grande do Sul, Brazil.

The initial examination was based on the observation of characteristic pure colonies under a light microscope. Then, microscope slides were prepared from the formed mycelium, stained with modified Amann's blue stain, and observed under a light microscope to confirm the identity of the fungi at the genus level [27,28].

For DNA extraction, fungal material from each isolate was collected and ground into a fine powder in liquid nitrogen using a pestle and pre-chilled 1.5 mL microtubes. Total genomic DNA was extracted from 100 mg of the homogenized material using the Wizard® Genomic DNA Purification Kit, following the manufacturer's instructions. After extraction, the DNA concentration was quantified using a spectrophotometer by measuring absorbance at 260 nm and 280 nm. The DNA concentration was adjusted to 50 $ng \cdot \mu L^{-1}$ using ultrapure water.

The molecular identity of the isolates was determined through PCR amplification and sequencing of the internal transcribed spacer (ITS) regions of ribosomal DNA, elongation factor 1-alpha (EF), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and the second largest subunit of DNA-dependent RNA polymerase II (RPB2) using the following primers: V9G-F (5′-TTACGTCCCTGCCCTTTGTA-3′) and LR5-R (5′-ATCCTGAGGGAAACTTC-3′) [29,30]; EF1-728F (5′-CATCGAGAAGTTCGAGAAGG-3′) and EF1-986R (5′-TACTTGAAGGAACCCTTACC-3′) [31]; GDF (5′-GCCGTCAACGACCCCTTCATTGA-3′) and GDR (5′-GGGTGGAGTCGTACTTGAGCATGT-3′) [31]; 7cR (5′-CCCATRGCTTGYTTRCCCAT-3′) and 5F2 (5′-GGGGWGAYCAGAAGAAGGC-3′) [32]. PCR reaction mixtures and thermal cycling conditions for each gene are summarized in Table 1.

The PCR product was subjected to electrophoresis on a 1.5% (w/v) agarose gel prepared with 0.5× Tris–Borate–EDTA (TBE) buffer, run at 90 V for 80 min. A loading buffer containing blue-green dye (LGC) was used, and bands were visualized under ultraviolet light using a gel imaging system (Safe Imager Blue Light Transilluminator, Invitrogen). Fragment size was estimated by comparison with a 1 kb molecular weight marker.

PCR products were purified using the ReliaPrep™ DNA Clean-Up and Concentration System. Sequencing was performed in both directions using the same PCR primers by ATCGene molecular analysis (Porto Alegre, RS, Brazil).

The resulting sequences were edited using BioEdit software (version 7.0.5.3). Consensus sequences were analyzed with molecular evolutionary genetics analysis (MEGA X, version 10.2.6) software [33], aligned using the MUSCLE algorithm, and compared with sequences from the NCBI GenBank database. Sequence similarity for ITS, EF, GAPDH, and RPB2 genes was assessed using the Basic Local Alignment Search Tool (BLAST, version 2.14.0). Phylogenetic analyses were conducted using the maximum likelihood method in MEGA X with 1000 bootstrap replicates. Reference sequences corresponding to each gene, previously deposited in GenBank, were also included in the analyses.

Table 1. Composition of PCR reaction mixtures (total volume 15 µL) and thermal cycling conditions used for the amplification of ITS, EF, GAPDH, and RPB2 genes.

Component/Condition	ITS	EF	GAPDH	RPB2
Total Volume (µL)	15	15	15	15
Genomic DNA (µL)	2.0	2.0	2.0	2.0
Primer (µL each)	0.30	0.15	0.15	0.30
Ultrapure Water (µL)	9.88	9.43	10.18	10.03
Taq Polymerase (µL)	0.12	0.12	0.12	0.12
10× PCR Buffer (µL)	1.50	1.50	1.50	1.50
$MgCl_2$ (µL)	0.6	0.6	0.6	0.45
DMSO (µL)	–	0.75	–	–
dNTPs (µL)	0.3	0.3	0.3	0.3
Initial Denaturation	95 °C 5 min	94 °C 1 min	94 °C 4 min	94 °C 1 min 30 s
Denaturation	94 °C 30 s	94 °C 30 s	94 °C 45 s	94 °C 30 s

Detailed composition of PCR mixtures and cycling conditions for amplification of fungal genes ITS, EF, GAPDH, and RPB2. The total reaction volume was 15 µL, including genomic DNA, primers (25 µmol·L^{-1}), ultrapure water, Taq DNA polymerase, 10× PCR buffer, $MgCl_2$, DMSO (when used), and dNTPs. Thermal cycling conditions include initial denaturation, denaturation, annealing, extension, and final extension steps with respective temperatures and durations.

2.3. Characterization of Spirulina platensis Biomass and Extract Production

To determine the protein and carbohydrate concentrations of *S. platensis*, the biomass was suspended at a concentration of 0.5 mg·mL^{-1} and subjected to sonication using an ultrasound probe for 10 min (1 min on, 1 min off) at maximum power [24]. Protein content was determined using the method of Lowry et al. (1951) [34], based on a bovine serum albumin standard curve. Carbohydrate content was quantified using the method of DuBois et al. (1956) [35], based on a standard curve prepared with anhydrous glucose.

S. platensis extracts prepared using different methods were produced at a concentration of 1% (*w*/*v*) for those involving phosphate buffer (pH 7.0, 0.15 mol·L^{-1}), which was prepared using monobasic sodium phosphate (NaH_2PO_4) and dibasic sodium phosphate (Na_2HPO_4) salts [22] and 5% for treatments using hydroalcoholic extraction (1:1 absolute ethyl alcohol and distilled water) [21]. For better homogenization, the extracts were subjected to Ultra-Turrax at 25,000 rpm for 5 min. After this initial homogenization, the extracts were further processed using either an ultrasonic probe (5 min at 100% power) [24] or an ultrasonic bath (37 Hz, 30 min at 30 °C) [25]. Table 2 presents the extraction methods used. All treatments were performed in triplicate.

The extracts obtained using the methodologies described in Table 2 were evaluated for their PC and TPC contents. Hydroalcoholic extracts (C, C+UP, and C+UB) were evaporated at 77 °C using a rotary evaporator to remove ethanol and subsequently resuspended in water to reach the initial concentration (5% *w*/*v*) [21].

To determine the PC concentration, the extracts were centrifuged (3600× *g* for 15 min) and then diluted in water to ensure that the absorbance remained between 0.200 and 0.800. Absorbance was measured at wavelengths of 615 and 652 nm using a spectrophotometer. The PC concentration was calculated using Equation (1) proposed by Bennett and Bogorad (1973) [37] and expressed as mg of PC per gram of *S. platensis*:

$$PC = [A_{620} - (0.474 \times A_{652})] \div 5.34 \quad (1)$$

where

PC = phycocyanin concentration ($mg \cdot mL^{-1}$);
A_{620} = absorbance at 620 nm;
A_{652} = absorbance at 652 nm.

Table 2. Methods used for the extraction of compounds from *Spirulina platensis* biomass and their abbreviations.

Extraction Type	Abbreviation	References
1% (*w*/*v*) biomass homogenized in phosphate buffer for 21 h at 4 °C	A	[22]
1% (*w*/*v*) biomass homogenized in phosphate buffer for 21 h at 4 °C, followed by ultrasonic bath treatment	A+UB [1]	[22,25]
1% (*w*/*v*) biomass homogenized in phosphate buffer for 21 h at 4 °C, followed by ultrasound probe treatment	A+UP [2]	[22,24]
1% (*w*/*v*) biomass homogenized in phosphate buffer for 4 h at 25 °C	B	[22,36]
1% (*w*/*v*) biomass homogenized in phosphate buffer for 4 h at 25 °C, followed by ultrasonic bath treatment	B+UB	[22,25,36]
1% (*w*/*v*) biomass homogenized in phosphate buffer for 4 h at 25 °C, followed by ultrasound probe treatment	B+UP	[22,24,36]
5% (*w*/*v*) biomass homogenized in hydroalcoholic solvent, followed by shaking (150 rpm) for 60 min at 25 °C	C	[21,25]
5% (*w*/*v*) biomass homogenized in hydroalcoholic solvent, followed by ultrasonic bath treatment, and then shaking (150 rpm) for 60 min	C+UB	[21,25]
5% (*w*/*v*) biomass homogenized in hydroalcoholic solvent, followed by ultrasound probe treatment, and then shaking (150 rpm) for 60 min at 25 °C	C+UP	[21,24]

[1] ultrasonic bath treatment; [2] ultrasound probe treatment.

For TPC analysis, the extracts were purified with hexane to remove nonpolar compounds. The lower phase was treated with barium hydroxide (0.1 mol L^{-1}) and zinc sulfate heptahydrate (5% *w*/*v*) to eliminate interfering substances [38]. The purified extracts were then centrifuged (3600× *g* for 15 min).

TPC was quantified based on a redox reaction with the Folin–Ciocalteu reagent, which reacts with hydroxyl groups in polyphenols. The procedure followed the method described by Correia et al. (2004) [39], with modifications proposed by Sousa and Correia (2012) [40]. Absorbance was measured at 765 nm using a spectrophotometer (UV-1600, Pró-Tools, Brazil). A calibration curve was previously established using gallic acid equivalents (GAE) as the standard ($Y = 8.461X - 0.1386$), with a determination coefficient (R^2) of 0.9977. Results were expressed as a GAE per gram of *S. platensis*.

After identifying the extraction method that resulted in the highest contents of PC and TPC, extracts were prepared at concentrations of 5, 10, and 15% (*w*/*v*) to study the effect of increasing the initial biomass concentration. The extracts were prepared using the A+UP and B+UP methods (Table 2). The abbreviations assigned to these extracts were:

A+UP.5%, A+UP.10%, A+UP.15%, B+UP.5%, B+UP.10%, and B+UP.15%. All treatments were performed in triplicate.

2.4. In Vitro Assay for Phytopathogen Inhibition

For the in vitro control assays, the phytopathogens *Colletotrichum orchidearum* 37083, *Fusarium nirenbergiae* 37084, and *Alternaria* sp. 37085 were used, previously isolated as described in Section 2.2. The *S. platensis* extract used in the tests was obtained under the best experimental condition identified in Section 2.3. To prevent contamination during the in vitro assay, the extract was subjected to two sterilization methods (autoclaving at 121 °C for 20 min and UV light exposure for 1 h) and was then characterized for PC and TPC.

The treatments tested were as follows: PDA (Kasvi, Brazil) medium without extract, used as a negative control (C1); PDA supplemented with phosphate buffer (0.15 mol·L^{-1}, pH 7.0), autoclaved at 121 °C for 20 min, representing the solvent control used in the extraction procedures (C2); PDA supplemented with *S. platensis* extract at 10% concentration, previously autoclaved under the same conditions as control C2 (A+UP.10%Autoclave); and PDA supplemented with *S. platensis* extract at 10% concentration, sterilized by UV light exposure for 1 h (A+UP.10%UV).

The assay consisted of applying 100 µL of each treatment onto 30 mL of PDA medium in 100 × 20 mm Petri dishes [41]. Treatments were pipetted and evenly spread using a Drigalski spatula. For treatment A+UP.10%UV, after the extract was applied to the plates, they were kept open under UV light for 1 h.

After complete absorption of the biocontrol agents, a 5 mm mycelial disc from each fungal colony isolated was placed at the center of the plate. Mycelial growth (mm) of each colony was measured along orthogonal axes on days 1, 3, 5, and 7 of incubation. Radius measurements were converted into areas using the equation $A = \pi r^2$; inhibition percentages were calculated. All treatments were conducted in quadruplicate, representing technical replicates of the same isolate.

The percentage of inhibition (PI) of the pathogens was determined using Equation (2), which considers the difference in colony diameter in the presence of biocontrol agents compared to the control [42]. For the calculations, the control used in the equation was C2 (Table 2). PI was calculated using the average colony area of the replicates for each day, and then the mean inhibition was obtained as follows:

$$PI = [(PC - PE) \div PC] \times 100 \quad (2)$$

where

PC = pathogen growth on the control plate (mm);
PE = pathogen growth on the plate containing biocontrol agents (mm).

2.5. Data Processing and Statistical Analysis

This section includes all statistical analyses performed in the manuscript, including pathogen growth assays, yields of bioactive compound extractions, and other evaluations. When necessary, data were transformed using the Box-Cox method (when applied, the λ value is reported in Section 3). Most analyses were conducted using the ExpDes.pt package (version 1.2.2) in R [43], which provides tools for experimental design and analysis in Portuguese. The Shapiro–Wilk test was used to assess the normality of residuals ($p > 0.05$); Bartlett's test was used to assess the homogeneity of variances ($p > 0.05$). For the analysis of inhibition percentages over time, a completely randomized design in a factorial scheme (two factors: treatment and time) was adopted. These analyses were performed using

Statistica software, version 10 (StatSoft Inc., USA). Phylogenetic analysis was performed using MEGA X molecular evolutionary genetics analysis across computing platforms [33].

3. Results

3.1. Identification of Phytopathogens

The images illustrating the fungal growth characteristics on the plates are shown in Figure 1. Figure 1a,d,g shows the fungus *Alternaria* sp. 37085, a phytopathogen that reproduces exclusively through conidia formed at the tips of conidiophores [14]. It exhibits mycelial growth with coloration ranging from pale to olive brown, a smooth to slightly verrucose surface, and septate conidia [44,45]. The conidia observed in the images conform to the described morphological pattern, forming branched chains. They display obclavate, ellipsoidal, or oval shapes and are septate [44,45]. The phylogenetic clade of *Alternaria* sp. 37085 is shown in Figure 1g, where the isolate clustered with reference strains of *Alternaria* sp. based on ITS region primers and the maximum likelihood method (sequence available in Appendix A).

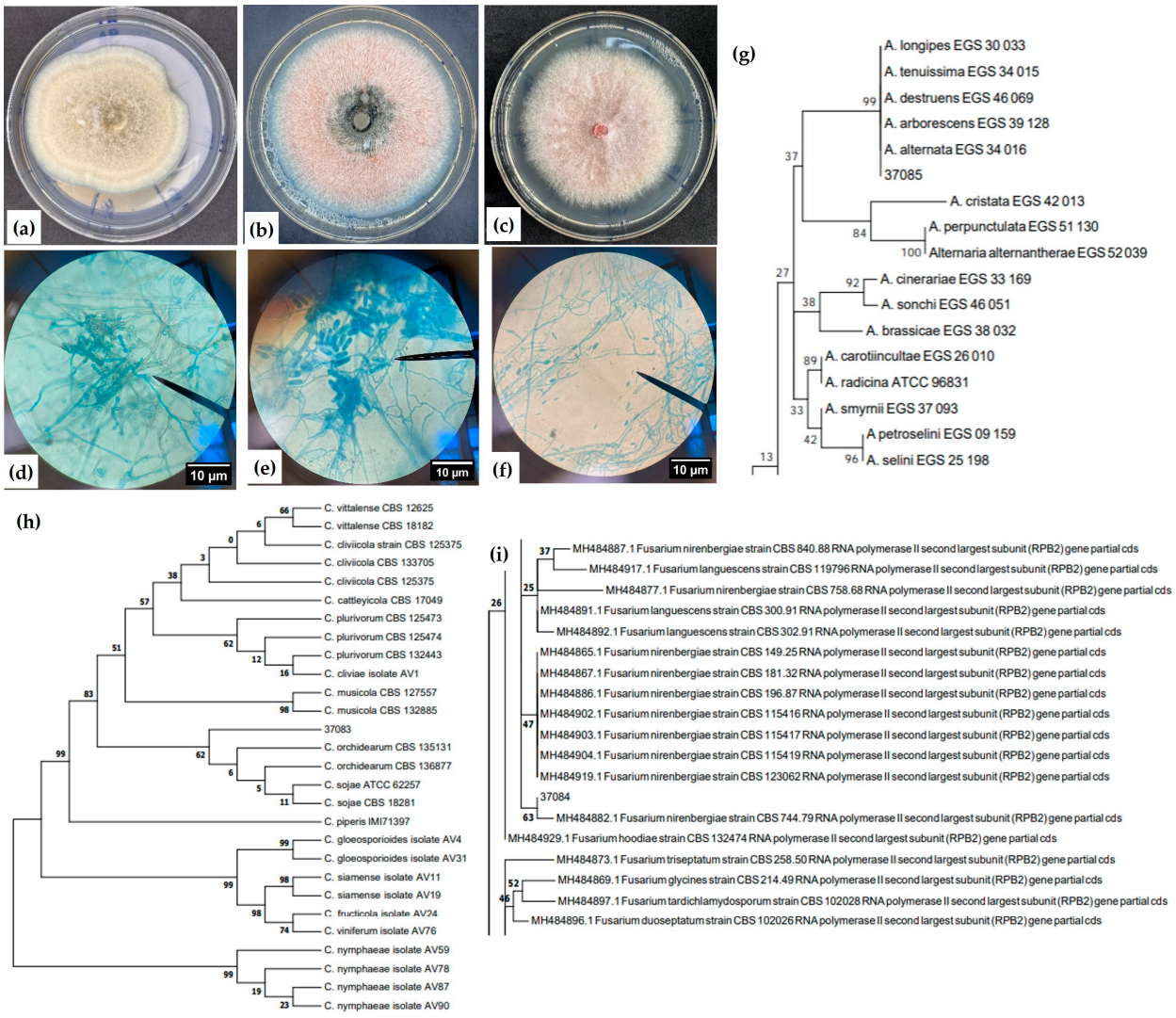


Figure 1. Macroscopic and microscopic structures (hyphae and spores) and phylogenetic clades of phytopathogenic fungi isolated from the roots of hydroponically grown lettuce: (**a**,**d**,**g**) *Alternaria* sp. 37085; (**b**,**e**,**h**) *Colletotrichum orchidearum* 37083; and (**c**,**f**,**i**) *Fusarium nirenbergiae* 37084.

Figure 1b,e,h refers to the phytopathogen *C. orchidearum* 37083. In the microscopic image, typical fungal structures can be identified such as smooth-walled conidia with a rounded apex. In the macroscopic image, pink fungal growth with black spots is observed; these small black structures are known as acervuli [14]. In Figure 1h, the isolate was grouped based on GAPDH (Appendix A) region primers and the maximum likelihood method, clustering with reference strains of *C. orchidearum*. *C. orchidearum* is a taxonomically recently described species, first identified in 2018, and belongs to the *C. gloeosporioides* species complex [15].

Figure 1c,f,i represents the phytopathogen *F. nirenbergiae* 37084. The macroscopic image shows a fungus with pink coloration and a velvety appearance, while the microscopic image reveals short monophialides with microconidia typical of this genus [46,47]. Phylogenetic analysis using RPB2 region primers and the maximum likelihood method showed that the isolate clustered with reference strains of *F. nirenbergiae* (Figure 1i). Additionally, BLAST (version 2.14.0) comparison of the EF (Appendix A) gene showed 100% similarity with the same species.

3.2. *Spirulina platensis* Biomass Characterization and Extract Yields

The biomass of *S. platensis* exhibited a high protein content (54.41 g·100 g^{-1}), while the carbohydrate content was 15.61 g·100 g^{-1}. The extraction methods had a significant effect ($p < 0.05$) on the extraction of PC and TPC.

The PC extraction is presented in Figure 2. The A+UP and B+UP methods resulted in the highest extraction yields (76 mg of PC extracted per gram of *S. platensis*), with no significant difference between them. Hydroalcoholic mixture-based methods (C, C+UB, and C+UP) led to significantly lower PC extraction yields from *S. platensis* biomass.

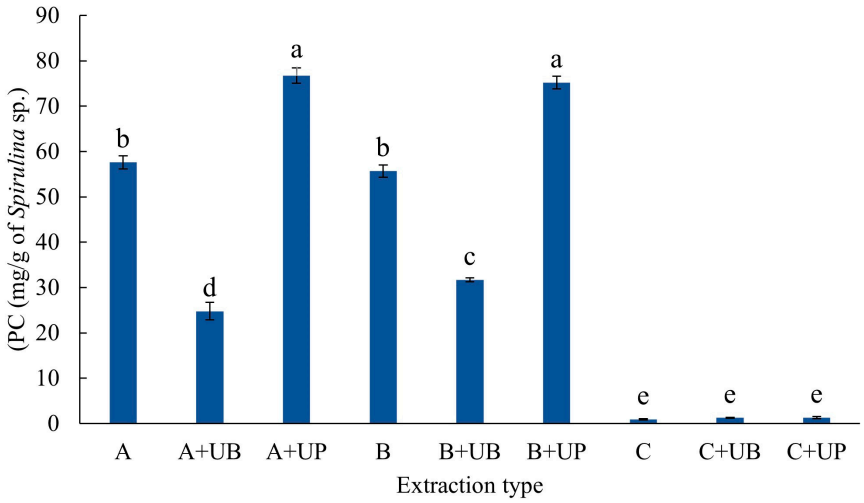


Figure 2. Phycocyanin (PC) concentration extracted from the microalga *Spirulina platensis* using each extraction method presented in Table 2. Treatments were as follows: A corresponds to 1% ($w{\cdot}v^{-1}$) biomass homogenized in phosphate buffer for 21 h at 4 °C. A+UB underwent the same procedure as A, followed by ultrasonic bath treatment, while A+UP was treated as A, followed by ultrasound probe treatment; B corresponds to 1% ($w{\cdot}v^{-1}$) biomass homogenized in phosphate buffer for 4 h at 25 °C; B+UB and B+UP followed the same initial procedure as B, followed by ultrasonic bath and ultrasound probe treatment, respectively; C refers to 5% (w/v) biomass homogenized in a hydroalcoholic solvent, followed by shaking at 150 rpm for 60 min at 25 °C.; C+UB was treated as C, followed by ultrasonic bath and subsequent shaking, while C+UP underwent the same as C, followed by ultrasound probe treatment and shaking at 150 rpm for 60 min at 25 °C. Data are expressed as means ± standard deviation ($n = 3$). Different letters indicate significant differences between treatments according to Tukey's test ($p < 0.05$). Data were transformed using the Box-Cox method ($\lambda = 0.2626$).

The extracts obtained using phosphate buffer, whether by homogenization (methods B and A) or by ultrasound (bath and probe), showed the highest concentrations of total TPC (20.95 mg GAE·g of *S. platensis*$^{-1}$), with no significant differences between them. In contrast, treatments using hydroalcoholic mixtures resulted in lower concentrations of TPC (Figure 3).

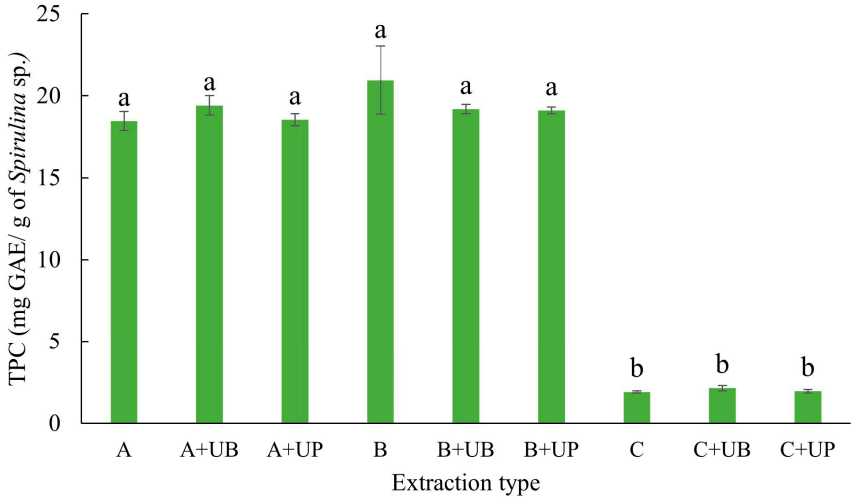


Figure 3. Total phenolic compound (TPC) concentration extracted from the microalga *Spirulina platensis* using each extraction method presented in Table 2. Treatments were as follows: A corresponds to 1% (*w*/*v*) biomass homogenized in phosphate buffer for 21 h at 4 °C. A+UB underwent the same procedure as A, followed by ultrasonic bath treatment, while A+UP was treated as A, followed by ultrasound probe treatment; B corresponds to 1% (*w*/*v*) biomass homogenized in phosphate buffer for 4 h at 25 °C; B+UB and B+UP followed the same initial procedure as B, followed by ultrasonic bath and ultrasound probe treatment, respectively; C refers to 5% (*w*/*v*) biomass homogenized in a hydroalcoholic solvent, followed by shaking at 150 rpm for 60 min at 25 °C.; C+UB was treated as C, followed by ultrasonic bath and subsequent shaking, while C+UP underwent the same as C, followed by ultrasound probe treatment and shaking at 150 rpm for 60 min at 25 °C. Data are expressed as means ± standard deviation (n = 3). Different letters indicate significant differences between treatments according to Tukey's test ($p < 0.05$).

The extracted concentrations of TPC (20.95 mg GAE·g^{-1}) and PC (76 mg PC·g^{-1}) using phosphate buffer (0.15 mol·L^{-1}, pH 7.0) followed by sonication after 21 or 4 h of extraction (methods A+UP and B+UP, respectively) stood out in our study. Sonication with an ultrasonic probe was particularly effective, resulting in the highest yields for both target compounds.

The ultrasonic probe is known for its ability to disrupt microalgal cell walls, efficiently releasing intracellular compounds [24,25]. This is consistent with the results obtained in our study, where ultrasonic treatments significantly enhanced extraction efficiency (Figures 2 and 3). During the process, ultrasonic waves generate microcavitation in the liquid medium. The collapse of these bubbles causes mechanical rupture of the cells, facilitating the release of TPC and other bioactive metabolites [21]. Thus, the combination of phosphate buffer and sonication enhances the extraction efficiency, improving both the yield and quality of the extracted compounds.

Based on the results obtained in the previous stage, extractions were performed using phosphate buffer (0.15 mol·L^{-1}, pH 7.0) at three different concentrations—5, 10, and 15% (*w*/*v*)—using the A+UP and B+UP treatments. The two-way analysis of variance was significant ($p < 0.05$). The mean comparisons analyzed by Tukey's test are presented in Figure 4a for PC and Figure 4b for TPC.

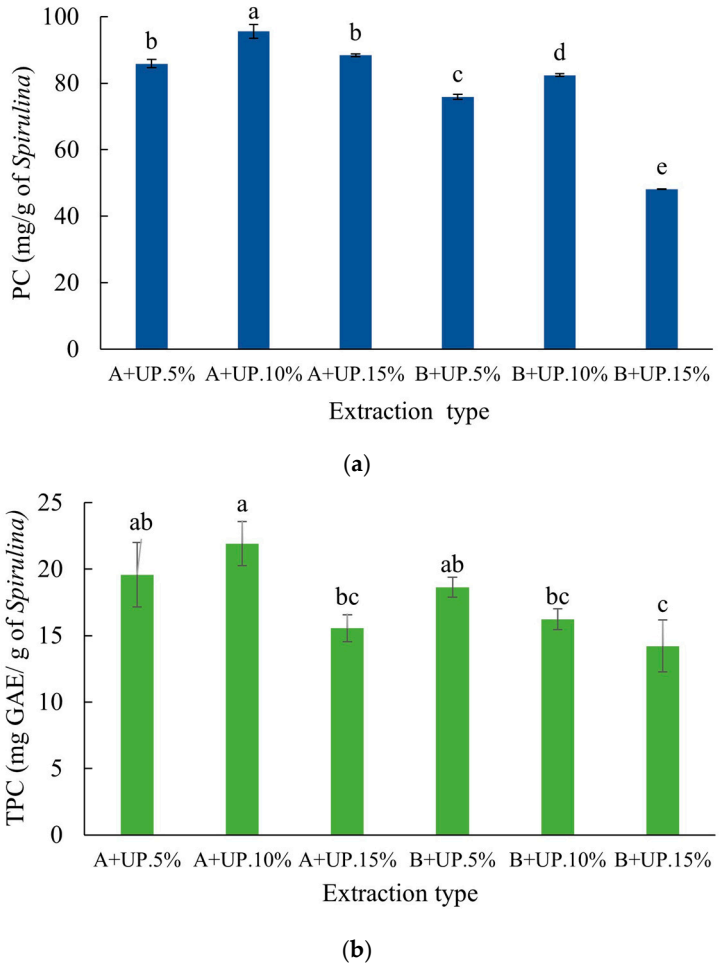
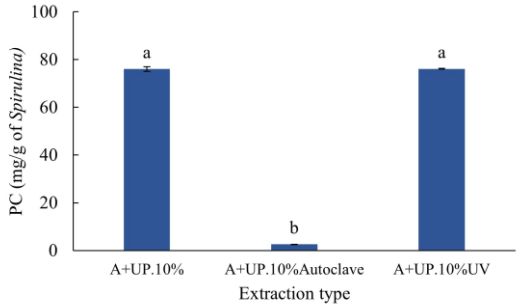


Figure 4. Phycocyanin (PC) and total phenolic content (TPC) of sonicated *Spirulina platensis* extracts at different concentrations (5, 10, and 15% *w*/*v*): (**a**) phycocyanin (PC); and (**b**) total phenolic content (TPC). Treatment A+UP corresponds to biomass homogenized in phosphate buffer for 21 h at 4 °C, and treatment B+UP corresponds to biomass homogenized in phosphate buffer for 4 h at 25 °C. All extracts were subjected to ultrasound probe treatment after the extraction period in buffer solution. Extracts were formulated at concentrations of 5, 10, and 15% (*w*/*v*). Data are expressed as means ± standard deviation (n = 3). Different letters indicate significant differences between treatments according to Tukey's test ($p < 0.05$).

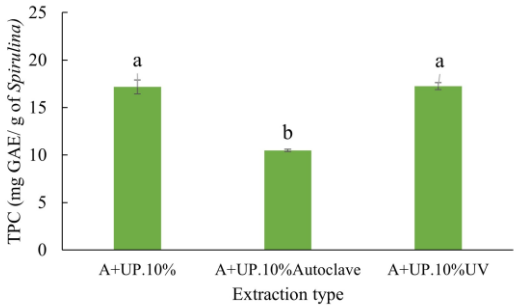
The graphical representations of PC (Figure 4a) and TPC (Figure 4b) extractions show that the A+UP.10% method resulted in the highest yields, reaching 95.6 mg g^{-1} for PC and 21.9 mg GAE·g^{-1} for TPC. Using the same biomass concentration, the B+UP 10% method yielded significantly lower amounts for both compounds. Additionally, the A+UP 15% method outperformed B+UP 15% in the PC extraction, indicating that longer extraction times favor PC release. However, extracts obtained with 15% biomass showed lower values than those with 10%, suggesting that higher concentrations may lead to suspension saturation, hindering pigment release. Under such conditions, the extracting solvent has less individual access to the cells, limiting PC diffusion and resulting in incomplete extraction and a lower yield per gram of biomass [48]. These findings demonstrate that both biomass concentration and extraction time significantly influence the extraction efficiency of PC and TPC from *S. platensis*, supporting previous studies that emphasize the importance of balancing these factors to optimize the recovery of proteins and pigments from cyanobacteria [25].

The *S. platensis* extract used for the in vitro control assays was prepared using 10% biomass and 21 h of extraction in phosphate buffer (0.15 mol·L^{-1}, pH 7.0) at 4 °C, in the dark.

The analysis of variance regarding the effect of the sterilization method (autoclaving and UV light) of the PC and TPC extraction was significant ($p < 0.05$). The mean comparisons are presented in Figures 5a and 5b, respectively.



(a)



(b)

Figure 5. Phycocyanin (PC) and total phenolic compounds (TPC) in *Spirulina platensis* extracts after different sterilization methods: (**a**) PC; and (**b**) TPC. The treatment A+UP.10% refers to the *S. platensis* extract at a concentration of 10% (*w*/*v*), obtained by extraction in phosphate buffer for 21 h at 4 °C, followed by ultrasound probe sonication without any sterilization method. A+UP.10%Autoclave refers to the same extract sterilized by autoclaving, and A+UP.10%UV refers to the extract sterilized by UV light for 1 h. Data are expressed as means ± standard deviation ($n = 3$). Different letters indicate significant differences between treatments according to Tukey's test ($p < 0.05$).

The non-sterilized extract (A+UP.10%) and the extract exposed to UV light for one hour (A+UP.10%UV) both showed 76 mg of PC·g^{-1} of *S. platensis*, with no significant difference between them (Figure 5a). However, the extract subjected to autoclaving (A+UP.10%Autoclave) showed a significant reduction in PC concentration, resulting in 2.62 mg PC·g^{-1} of *S. platensis*. Regarding TPC content, both extracts A+UP.10% and A+UP.10%UV exhibited statistically sim ilar values (17.25 mg GAE.g *S. platensis*$^{-1}$) (Figure 5b). In contrast, the autoclaved extract showed a significant reduction in TPC content, reaching 10.49 mg GAE·(g of *S. platensis*)$^{-1}$.

3.3. Efficacy of Extracts in Controlling Phytopathogens In Vitro

Figure 6 presents the antifungal activities against the isolated phytopathogens. Figure 6a–c refer to *C. orchidearum*, Figure 6d–f to *F. nirenbergiae*, and Figure 6g–i to *Alternaria* sp. Panels a, d, and g show the inhibition percentages over seven days. Panels b, e, and h depict the radial growth progression; and panels c, f, and i show the fungal growth on day seven.

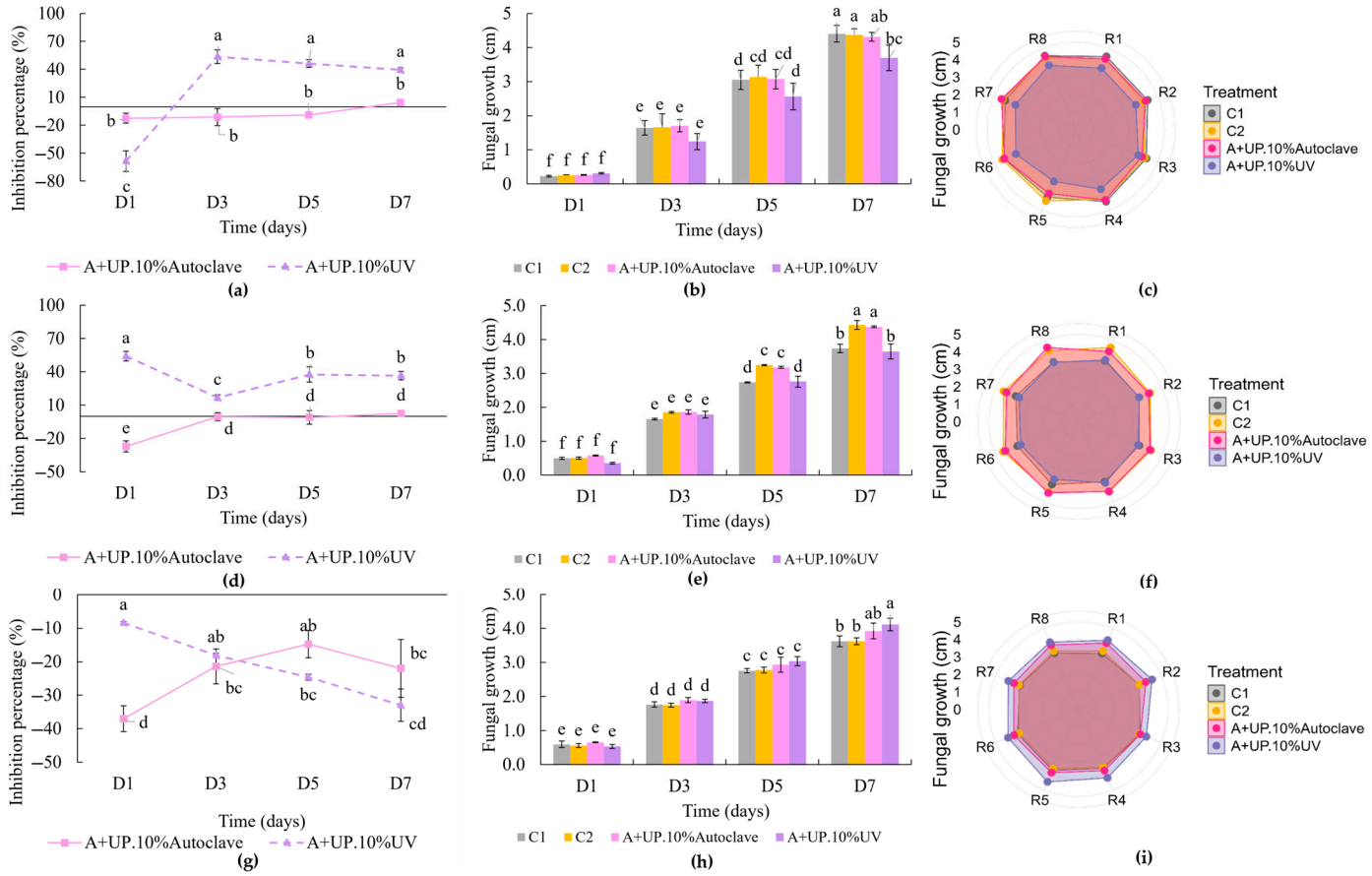


Figure 6. Inhibition percentages of phytopathogens over seven days of treatment with *Spirulina platensis* extracts, fungal radial growth over time, and radial visual representation of phytopathogens on the seventh day: (**a**–**c**) *Colletotrichum orchidearum* 37083; (**d**–**f**) *Fusarium nirenbergiae* 37084; and (**g**–**i**) *Alternaria* sp. 37085 in response to controls and *S. platensis* extracts. Figures (**a**,**d**,**g**) show the inhibition percentages resulting from extract treatments after subtracting the phosphate buffer control (C2), while figures (**c**,**f**,**i**) show the radial growth of the fungi in response to the extracts compared to the controls. Figures (**b**,**e**,**h**) illustrate the radial growth of the phytopathogens throughout the seven-day period. Treatment A+UP.10%Autoclave refers to the *S. platensis* extract at 10% (*w*/*v*), extracted in phosphate buffer for 21 h at 4 °C, sonicated with an ultrasound probe, and sterilized by autoclaving. Treatment A+UP.10%UV refers to the same extract sterilized by UV light for 1 h. Data are expressed as means ± standard deviation (n = 4). Means followed by the same letter do not differ significantly according to Tukey's test at 5% probability.

The antifphenoungal effects observed in this study reinforce the idea that the activity of microalgal extracts is highly specific and depends on the combination between the target microorganism and the type of microalga used [49]. For the phytopathogen *C. orchidearum* (Figure 6a), the A+UP.10%UV treatment showed the highest inhibition on the third day of the experiment, reaching 53.52% inhibition, with no significant variation observed until the end of the trial. Although the radial growth trends over time shown in Figure 6b do not reveal a clear separation between treatments and controls throughout the entire experimental period, a marked difference becomes evident on the seventh day. This is further supported by Figure 6c, which shows the final colony diameters and highlights the pronounced inhibitory effect of the A+UP.10%UV treatment.

The performance of the extracts on the inhibition of the phytopathogen *F. nirenbergiae* 37084 is shown in Figure 6d. The A+UP.10%UV extract exhibited the highest inhibition, with the best result observed on the first day of the experiment (54.17%), showing no statistical difference compared to the seventh day. Both the A+UP.10%UV extract and the

control C1 (PDA only) showed lower mycelial growth than the other treatments starting from the fifth day, as shown in Figure 6e, suggesting that the phosphate buffer facilitated fungal growth. Fungal growth on the seventh day can be observed in Figure 6f. Similar to the previous phytopathogen, radial growth was reduced following the application of the A+UP.10%UV extract. Treatments involving the autoclaved extract (A+UP.10%Autoclave) showed much lower effectiveness, with the highest inhibition recorded on the seventh day at only 2.51%.

Figure 6g–i shows the inhibition levels of the extracts against the phytopathogen *Alternaria* sp. The treatments resulted in fungal growth promotion, which is the opposite effect compared to the other phytopathogens studied. The lowest inhibition percentages were observed with the A+UP.10%Autoclave extract on the first day (−37.02%), while the A+UP.10%UV extract resulted in −32.96% inhibition. The ineffectiveness of the extracts against *Alternaria* sp. is also evident in Figure 6h, where mycelial growth was higher in the treatments compared to the controls on the seventh day of the experiment. This can also be visually observed in the radial representation (Figure 6i). These results suggest a potential fungal growth-promoting effect of the extracts against *Alternaria* sp.

4. Discussion

The phytopathogens *C. orchidearum*, *F. nirenbergiae*, and *Alternaria* sp., isolated in this study from the roots of hydroponically grown lettuce, are known to cause diseases in various plant species, significantly affecting crop yields. Their identification in lettuce underscores the growing phytosanitary challenges associated with soilless cultivation systems such as hydroponics.

Notably, *C. orchidearum* was identified for the first time in lettuce. To the best of our knowledge, there are no prior reports in the literature of this species being isolated from lettuce. *C. orchidearum* is a recently described species in taxonomic terms, primarily associated with ornamental plants, such as orchids (*Cattleya* sp., *Dendrobium* sp., *Oncidium* sp., and *Phalaenopsis* sp.), *Cordyline terminalis*, and *Monstera deliciosa*, with most records originating from Asian countries [13,14,50]. Its detection in this study extends the known host range of the species and represents the first report of *C. orchidearum* associated with lettuce in Brazil, raising an important alert for hydroponic production systems.

The isolation of *F. nirenbergiae*, another recently described species (taxonomically) within the *F. oxysporum* species complex (FOSC), is also noteworthy. This pathogen has been associated with vascular wilts in crops such as passion fruit [11] and saffron [12]. Its occurrence in hydroponic lettuce highlights the need for preventive measures and regular monitoring, even in soilless systems, given that members of the FOSC are cosmopolitan, asexual, and often transmitted through plant debris or contaminated water.

The application of sonication, including ultrasonic bath and probe treatments, was effective in enhancing the extraction action of intracellular compounds such as phycocyanin. As this pigment is located within phycobilisomes attached to thylakoid membranes [51], cell wall disruption is necessary for its efficient release. Ultrasonic probe treatment facilitates this through acoustic cavitation, generating localized high pressures and shear forces that rupture cells [24,25]. Importantly, shorter extraction times at room temperature yielded PC amounts comparable to those obtained under longer, colder conditions, indicating a potentially more efficient and economical process.

TPC are structurally diverse secondary metabolites found in microalgae, including phenolic acids, flavonoids, and tannins [51,52]. Their concentration in *S. platensis* biomass is known to vary depending on environmental and cultivation factors. These findings reinforce the importance of selecting appropriate extraction conditions. When using phosphate buffer combined with ultrasonication, higher TPC yields were observed compared to

hydroalcoholic extraction methods, indicating that the former may be more suitable for recovering phenolics from *S. platensis* [23]. This observation aligns with previous studies highlighting the enhanced efficiency of sonication-based methods when optimized for solvent composition and extraction parameters [21].

A comparative summary of extraction conditions and yields for PC and TPC, including data from this study and previous publications, is presented in Table 3 to enhance clarity and facilitate comparison.

Table 3. Summary of phycocyanin and total phenolic compound extraction results from literature and current study.

Study/Source	Extraction Method	Time/Conditions	Compound and Concentration
Brião et al. (2020) [4]	Phosphate buffer	21 h, 4 °C	58.54 mg·(g dry biomass)$^{-1}$ of PC
Current study (ultrasonic bath + probe)	Phosphate buffer + ultrasound (bath and probe)	4 h, room temperature or 21 h, 4 °C, followed by ultrasonic bath treatment (37 kHz, 30 min at 30 °C; P60H), or ultrasound probe treatment (5 min at 100% power).	~76 mg·(g dry biomass)$^{-1}$ of PC
Tavakoli et al. (2021) [21]	Sonication with WE70 solvent (70% ethanol + 30% water)	30 min, 100 W	28.78 mg GAE·(g dry biomass)$^{-1}$ of TPC
Martí-Quijal et al. (2021) [23]	50% Ethanol	120 min, room temperature	6.04 mg GAE·(g dry biomass)$^{-1}$ of TPC
Current study (hydroalcoholic mixture)	Hydroalcoholic mixture (ethanol:water 1:1)—subjected to shaking, followed by either ultrasonic bath or ultrasound probe treatment	Shaking at 150 rpm for 60 min at 25 °C, followed by ultrasonic bath treatment (37 kHz, 30 min at 30 °C; P60H), or ultrasound probe treatment (5 min at 100% power).	2.13 mg GAE·(g dry biomass)$^{-1}$ of TPC
Current study (phosphate buffer + ultrasound)	Phosphate buffer + ultrasound (bath and probe)	21 h, 4 °C, followed by ultrasonic bath treatment (37 kHz, 30 min at 30 °C; P60H), or ultrasound probe treatment (5 min at 100% power).	20.95 mg GAE·(g dry biomass)$^{-1}$ of TPC

Phycocyanin stability is affected by multiple factors such as production method, storage, temperature, and pH [53]. The use of phosphate buffer at a pH of 7.0 with sonication illustrates the importance of mild extraction conditions to preserve compound integrity [21,22]. Our data indicate that simultaneous extraction of PC and TPC can be efficiently achieved using 10% biomass in 0.15 mol L^{-1} phosphate buffer (pH 7.0) at 4 °C for 21 h, in the dark, assisted by sonication—a process that is both technically and economically viable.

Regarding sterilization, autoclaving significantly reduced the stability of the bioactive compounds, likely due to the thermosensitive nature of PC, which is known to degrade substantially at temperatures as low as 70 °C [53]. Conversely, sterilization by ultraviolet (UV) radiation better preserved both PC and TPC. This finding supports the use of UV radiation as a suitable alternative to thermal sterilization, also ensuring extract sterility, as previously demonstrated in studies applying UV–C light to eliminate microbial contamination in *S. platensis* cultures [54].

The antifungal potential of microalgal extracts is supported by the literature. Scaglioni et al. (2019) [55] reported that crude extracts from *Nannochloropsis* sp. and *S. platensis* were more effective against the *Fusarium graminearum* complex than purified fractions. Additionally, TPC from *S. platensis* have been shown to reduce fungal biomass markers, such as ergosterol and glucosamine, and inhibit ergosterol synthesis in vitro [56].

Schmid et al. (2022) [49] evaluated microalgal extracts against several phytopathogens, including *Alternaria alternata*. Their results indicated that *Alternaria* was not inhibited by any extracts tested, including those from *Spirulina*, *Nannochloropsis*, and *Scenedesmus*, with

Spirulina extract even promoting growth of *Sclerotium rolfsii*. This underscores the species- and extract-specific nature of microalgal bioactivity and highlights the importance of testing each pathogen–extract combination individually.

In contrast, the *S. platensis* extract (A+UP.10%UV) in this study inhibited *Colletotrichum* spp. growth by up to 53%, demonstrating significant antifungal activity. Sacramento et al. (2025) [57] similarly reported fungistatic effects of *Conticribra weissflogii* against *Colletotrichum gloeosporioides* at a minimum inhibitory concentration of 1.5 mg/mL. However, while Sacramento's results were based on purified compounds, our study achieved substantial inhibition using crude extracts, highlighting the practical potential of *S. platensis* for sustainable fungal control.

These findings demonstrate the potential of microalgal extracts—especially those sterilized by UV radiation—as selective antifungal agents. The results emphasize that crude *S. platensis* extracts, still underexplored for antifungal activity, can inhibit fungal growth without the need to isolate specific compounds. Additionally, the growth-promoting effect observed on *Alternaria* sp. highlights the complexity of these interactions and the importance of evaluating each pathogen–extract combination individually. Altogether, our results provide a promising foundation for future in vivo studies and support the integration of *S. platensis* extracts into sustainable disease management strategies, particularly in hydroponic and soilless systems.

5. Conclusions

The extraction performed with phosphate buffer (0.15 mol·L^{-1}, pH 7.0) combined with sonication, using 10% biomass for 21 h at 4 °C, proved to be the most efficient condition for the simultaneous extraction of PC and TPC from *Spirulina platensis*. The obtained extracts demonstrated promising in vitro antifungal activity against the phytopathogens *Colletotrichum orchidearum* 37083 and *Fusarium nirenbergiae* 37084, with this being the first report of *C. orchidearum* isolation from lettuce crops in Brazil. This finding broadens the knowledge of the host range of this species and highlights the importance of efficient control of this pathogen in hydroponic systems.

Conversely, the extracts promoted the growth of the phytopathogen *Alternaria* sp., indicating that the antifungal activity is highly specific and underscoring the need for detailed evaluations for practical applications. These results suggest that *S. platensis* algal extracts represent a viable and sustainable alternative for the biological management of *C. orchidearum* and *F. nirenbergiae* in agricultural systems.

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Data Availability Statement: Data are contained within the article.

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Conflicts of Interest: The authors declare no conflicts of interest.

Appendix A

Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene sequences
Sample 37083
CCCTTCATTGAGACCAAGTACGCTGTGAGTAGCACCCCTCCAAGCTCGCCGCGA
TATCACGCCCGCCACCCCTCAATCGCGAACGCCAGCTTCTGGCTGCCGATCAGAC
GCCAAAATCAATCAGGCTCTGATACAGCGAGCGATTGATGGGGCCGGCGCGGCG
GGGTCGAACATAGCCTCAATGGTTTCGGTTGCTGATACGCCATCCGCAGGCCTAC
ATGCTCAAGTACGACTC

Elongation factor (EF) gene sequences
Sample 37084
CACGGTGACCGGGAGCGTCTGAGTGATGTTAGTACGAAGAGAAGTAGAATGAAG
CATGAGCGACAACATACCAATGACGGTGACATAGTAGCGAGGAGTCTCGAACTT
CCAGAGAGCAATATCGATGGTGATACCACGCTCACGCTCGGCCTTGAGCTTGTCA
AGAACCCAGGCGTACTTGAAGGAACCCTTACCGAGCTCAGCGGCTTCCTATTGTT
GAATGGTTAGTGACTGCTTGACACGTGACGACGCACTCATTGAGGTTGTGAGAAT
GGTTAAGAGGGCAAACGCTCCCGTCGCTCAAGTGGCGGGGTAAGTGCCCCACCA
AAAAAAATTACAGTCATATTGCAAAATTTTTGGTCTCGAGCGGGGTAGCGGGCAC
GTTTCGAGTCGTAGGGGAAATCGATGGGCAAAGGACGCGCGATCGAAGGGAAA
GTGACTAACCTTCTCGAACTTCTCGATGGTTCGCTTATCGATACCACCGCACTGGT
AGATCAAGTGACCGGTCTGTGAAACGATGTCAGTATGTTGACTTTGAGAAATACC
CCACCAGGTCTTGGTCGGGATTGACGATGGCAGATAAGCTCATTGTCGAGGAGA
GTACTCACAGTG

Second largest subunit of DNA-directed RNA polymerase II (RPB2) gene sequences
Sample 37084
TCTTTCACATTTGCGTCGAACCAATACTCCCATCGGACGAGATGGTAAATTGGCCAAG
CCTCGACAGCTTCACAACACTCACTGGGGTTTGGTGTGTCCTGCCGAAACACCTGAG
GGTCAAGCTTGTGGTCTGGTCAAAAACTTGTCTCTGATGTGTTACGTCAGTGTCGGCT
CTCCAGCCGATCCTCTGATTGAATTCATGATCAACAGAGGTATGGAAGTCGTTGAGG
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GGATGGCTTGATCCGCGAAGGAGCTGTCGAGTATCTCGATGCTGAGGAAGAAGAGAC
AGCCATGATTTGCATGACGCCAGAGGATCTCGAACTTTACCGTGAGCAAAAGAATGA
TGAAGCTACACTCACAGAAGAAGAGAAACGGGCCAAGCAAGAGGCAGAGAAGAGA
GAACAAGAGGAGGACCGCAACAAGCGATTGAAGACAAAGGTGAACCCCACAACTC
ACATGTACACACATTGTGAGATTCACCCCAGTATGATTCTCGGTATCTGTGCCAGTAT
CATTCCTTTCCCCGATCACAACCAGGTATGTATGTCCTATGACC

Internal transcribed spacer (ITS) region sequences
Sample 37085
GCAACGACCACCTCAAGCCGGAAAGTTCGTCAAACTCGGTCATTTAGAGGAAGT
AAAAGTCGTAACAAGGTCTCCGTAGGTGAACCTGCGGAGGGATCATTACACAAA
TATGAAGGCGGGCTGGAACCTCTCGGGGTTACAGCCTTGCTGAATTATTCACCCT
TGTCTTTTGCGTACTTCTTGTTTCCTTGGTGGGTTCGCCCACCACTAGGACAAACA
TAAACCTTTTGTAATTGCAATCAGCGTCAGTAACAAATTAATAATTACAACTTTCA
ACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGCGATAAG
TAGTGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCC
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GCAGCCGGCCTACTGGTTTC

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