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Combined Effect of *Spirulina Platensis* and *Punica Granatum* Peel Extracts: Phytochemical Content and Antiphytopathogenic Activity

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Abstract: Biological control is one of the effective methods for managing plant diseases in food production and quality. In fact, there is a growing trend to find new bio-sources, such as marine algae and vegetal by-products. In this study, pomegranate (*Punica granatum*) peel (S1) and *Spirulina platensis* (S2) alone and in combinations, pomegranate peel/*Spirulina*: 25%/75% (S3) and 50%/50% (S4) were evaluated for antimycotoxigenic and antiphytopathogenic fungal properties. The chemical composition (moisture, dry matter, protein, lipid and ash) as well as total polyphenols, flavonoids and anthocyanins content were evaluated in the four extracts. Using agar diffusion and broth microdilution methods, the anti *Fusarium oxysporum*, *Fusarium culmorum*, *Fusarium graminearum*, *Aspergillus niger* and *Alternaria alternata* activities were measured and their correlations with phytochemical content were evaluated. Interestingly, combinations between *Spirulina* at 75% and pomegranate peel at 25% (S3) have a significant impact ($p < 0.05$) on the antifungal activity compared to S1, S2 and S4. These findings underlie the effectiveness of biocontrols over standard fungicides and imply that existing methods can be further improved by synergistic effects while maintaining food safety in an eco-friendly manner.

Keywords: *Spirulina*; pomegranate peel; antifungal activity; chemical composition; phytochemical content; antimycotoxigenic; antiphytopathogenic

1. Introduction

Due to the occurrence of fungal resistance to synthetic fungicides, the use of chemical compounds is strictly controlled and their application is subjected to tighter regulations [1,2]. Therefore, the use of biological substances with antimicrobial properties redirected researchers for the development of novel and stable approaches that are less inducible to antimicrobial resistance in order to replace chemical fungicides, bactericides and pesticides for food preservation and safety as well as plant crop protection. Furthermore, the growing interest in biological food additives has challenged the scientific community to innovate in alternative food preservation and plant crop protection systems. Thus,

natural preservatives from sources like bacteria, plants and algae were reported to ensure food safety due to their antimicrobial activity against a wide spectrum of foodborne pathogens [3,4]. In this regard, dietary antioxidants (i.e., phenolic compounds) have recently attracted extensive attention because of their ubiquity in nature and their various beneficial effects, including antimicrobial, antioxidant, antiinflammatory and antiproliferative activities [5].

One of the readily available sources of such compounds is by-products from the agro-food industry. For example, pomegranate processing generates a large amount of solid wastes. In fact, per 1000 g of raw pomegranate fruit, the edible portion accounts for only half (~400 g of juice and 100 g of seeds) and the rest, almost 500 g (e.g., peels), is discarded as waste [6]. Pomegranate peel has remarkable antioxidant and antimicrobial properties. Rich in flavonoids, phenolic acids and tannins, pomegranate peels are known to possess important and diverse biological and pharmacological properties [7,8]. Šavikin et al. reported that pomegranate peel exhibits high levels of free radical scavenging activities and strong antimicrobial activity due to its phenolic content [9]. Several studies reported the efficacy of extracts from pomegranate peel to inhibit both Gram-positive and Gram-negative bacterial growth such as foodborne pathogens, spoilage bacteria and human pathogens [10]. It was reported as well that pomegranate extracts display antifungal properties [11]. In fact, some extracts have anti *Candida* sp. and anti *Saccharomyces cerevisiae* activities [12] and, more recently, both peel powder and extracts were demonstrated to inhibit the growth of *Penicillium* spp., two mycotoxigenic *Aspergilli* strains (*A. flavus* and *A. ochraceus*) [13], two *Colletotricum* strains as well as *Rhizopus stolonifer*, *Botrytis cinerea*, and *Rhizoctonia solani* strains [14,15]. Some studies reported that the proportion of carbohydrate in pomegranate peel was about 15% and that the polysaccharides extracted from pomegranate had obvious biological functions [16].

Spirulina (*Arthrospira platensis*), a microscopic and filamentous cyanobacterium, has been recently recommended as a sustainable, highly nutritional and ecofriendly microalga [17]. *Spirulina* contains potent antioxidants, free-radical scavengers [18] and is able to inhibit the growth of some Gram-negative, Gram-positive bacteria and yeast such as *Candida albicans* [18]. Polysaccharides extracted from *Spirulina* have antitumor, antioxidation, antiaging, and antiviral properties [19]. Nevertheless, little information about antifungal properties, especially against mycotoxigenic and phytopathogenic fungi, of *Spirulina platensis* is available in the literature and its potential toxic effects have not been largely investigated.

To date, there is no study that has addressed mycotoxigenic and phytopathogenic properties when pomegranate and *Spirulina* were combined. Therefore, the objective of this research was to evaluate the composition of *Spirulina* alone and in combination with pomegranate peel extracts and to study their potential on the antifungal activity. Associations between phytochemical content and antifungal properties were discussed based on Pearson coefficients.

2. Materials and Methods

2.1. Algal Culture and Growth Conditions

Spirulina platensis was provided by Eden Life Aquaculture—Company (Gabes, Tunisia). Under photoautotrophic conditions, the culture was performed with Zarrouk medium containing NaHCO₃ (PubChem CID: 516892) 16.8 g; K₂HPO₄ (PubChem CID: 24450) 0.5 g; NaNO₃ (PubChem CID: 24268) 2.5 g; NaCl (PubChem CID: 5234) 1.0 g; MgSO₄ · 7H₂O (PubChem CID: 24843) 0.2 g; FeSO₄ · 7H₂O (PubChem CID: 24393) 0.01 g; K₂SO₄ (PubChem CID: 24507) 1.0 g; CaCl₂ · 2H₂O (PubChem CID: 5284359) 0.04 g and EDTA (PubChem CID: 6049) 0.08 g for 1 litre of medium [20]. Zarrouk's nutrient medium was purchased from Scharlau (Barcelona, Spain). The initial pH was adjusted to 9.5, and was found to increase only slightly during cultivation. The culture was illuminated at a light intensity of 80 mEm⁻²s⁻¹. The culture temperature was maintained at 30 ± 0.1 °C, while the culture was agitated at 300 rpm. *S. platensis* was grown for 108 h until the late exponential phase when cells were collected and freeze-dried.

2.2. Preparation of Ethanolic Extract of *Spirulina platensis* (*S. platensis*)

Ethanolic extract was prepared by stirring 100 g of freeze-dried *S. platensis* with 300 mL of ethanol (PubChem CID: 702) (Novachim, Bucharest, Romania) for 24 h at 40 °C in the dark. After filtration, the obtained extract was concentrated in a rotary evaporator (Laborota 4000, Heidolph, Milan, Italy), then dried in a lyophilizer (Martin Christ, Alpha 1-2 LD plus Germany). The resulting extract of *S. platensis* from ethanol was kept at +4 °C until use.

2.3. Plant Material and Extraction

Fruits of the pomegranate (*Punica granatum* L. Tunisian cultivar ‘Gabsi’) were obtained from a commercial harvest on local farms in Gabes (N: 33.53°, E: 10.07°). Peels were washed with running water, air-dried at room temperature, then powdered with an electric grinder (The original grinder, Moulinex, France) to the diameter of 0.5 mm. One hundred grams of powdered peels were soaked in ethanol at room temperature for 24 h in the dark. The extraction mixture was well decanted and filtered, then evaporated at 40 °C and lyophilized to obtain a yellow–brown residue which was immediately analyzed.

2.4. Extracts Preparation

The combination between pomegranate peel and *Spirulina* was assessed in order to detect the maximum synergistic effect between the two ethanolic extracts. Four samples: S1, S2, S3 and S4 representing the mixture design of its binary combinations: S1 (pomegranate peel), S2 (*Spirulina*), S3 (pomegranate peel—*Spirulina*: 25%/75%) and S4 (pomegranate peel—*Spirulina*: 50%/50%) were studied.

2.5. Physico-Chemical Analysis

2.5.1. Dry Matter

The dry matter was ascertained according to the Association of Official Analytical Chemists: samples were dried at 105 ± 3 °C to constant weights [21,22].

2.5.2. Protein Content

The total protein content was determined by the Kjeldahl method. The protein content was calculated by using a nitrogen conversion factor of 6.25 and expressed as percent of dry weight [22].

2.5.3. Fat Content

Lipids were extracted by using the protocol described in the standard NF V 03-713 [23]. Eight grams of each sample were hydrolyzed with a mixture of ethanol, formic acid (PubChem CID: 284) (Sigma, Taufkirchen, Germany) and 70% hydrochloric acid (PubChem CID: 313) (Sigma, Taufkirchen, Germany) for 20 min at 75 °C. Chloroform (PubChem CID: 6212) (Novachim, Bucharest, Romania) was then used as solvent for oil extraction and the samples were cooled under agitation. Sixteen milliliters of ethanol and 50 mL of chloroform were added and the mixtures obtained were agitated for 20 min. The extracting procedure was repeated twice. The collected solvent was then removed by means of rotary evaporator at 50 °C. These data were expressed as percentage of lipids per sample dry matter.

2.5.4. Ash Content

To remove carbon, each sample was ignited and incinerated in a muffle furnace at a temperature of 550 °C for 16 h [24].

2.6. Quantitative Determination of Phenolic Compounds

2.6.1. Total Polyphenols Content (TPC)

Total polyphenols content (TPC) of each sample was determined by the Folin–Ciocalteu method [25]. For each diluted extract, 10 microliters were mixed with 50 μ L of Folin–Ciocalteu reagent (Sigma-Aldrich GmbH, Steinheim, Germany) and shaken for 5 min. Then 150 μ L of 20% Na_2CO_3 (PubChem CID: 10340) (Scharlau, Barcelona, Spain) was added and the mixture was shaken once again for 1 min. Finally, the solution was set to 790 μ L by adding distilled water. After two hours, the absorbance at 760 nm was measured using a spectrophotometer (Bibby Scientific Limited, Stone, UK). The standard for the calibration curve was the Gallic acid (PubChem CID: 370) (Sigma-Aldrich GmbH, Steinheim, Germany) and finally TPC was expressed as mg of Gallic acid equivalent per g of sample (mg GAE/g) using the linear equation of the calibration curve.

2.6.2. Total Flavonoids Content (TFC)

Total flavonoids content (TFC) of each sample was determined using the method of Quettier-Deleu et al. [26]. One milliliter of AlCl_3 (PubChem CID: 24012) (Scharlau, Barcelona, Spain) was added to 1 mL of each diluted extract solution, vortexed and incubated for 15 min in the dark. The absorbance was evaluated at 430 nm and the quercetin (PubChem CID: 5280343) (Sigma-Aldrich GmbH, Steinheim, Germany) was used as standard for the calibration curve. TFC was expressed in mg of quercetin equivalent per g of extract (mg QE/g).

2.6.3. Total Tannins Content (TAC)

Total tannins content (TAC) was evaluated by the pH differential technique using 2 buffer systems: potassium chloride buffer pH1.0, 0.025 M (PubChem CID: 4873) (Sigma-Aldrich GmbH, Steinheim, Germany) and sodium acetate buffer 4.5, 0.4 M (PubChem CID: 517045) (Loba Chemie Pvt. Ltd., Mumbai, India) pH). Each extract was mixed with the corresponding buffer and read against water as blank in the two wavelength 510 and 700 nm [27]. Total tannins content was expressed as mg of cyanidin-3-glucoside equivalents (PubChem CID: 197081) (CGE) per 100 g of extract and was calculated from the following equation:

$$\text{TAC} = [(A_{510} - A_{700})_{\text{pH}1.0} - (A_{510} - A_{700})_{\text{pH}4.5} \times \text{MW} \times 1000] / \epsilon$$

where, A is absorbance value, M is molecular weight (449.2 g/mol), DF is dilution factor and Σ is the molar absorptivity of cyanidin3-glucoside (26,900 L/mol cm).

2.7. Antimicrobial Activities

2.7.1. Antifungal Assays

The antifungal activity of each extract was tested against *Fusarium oxysporum* CTM10402, *Aspergillus niger* CTM 10099, *Fusarium graminearum* ISPaVe 271, *Fusarium culmorum* ISPaVe 21w and *Alternaria alternata* CTM 10230. *Fusarium oxysporum*, *Aspergillus niger* and *Alternaria alternata* were provided by the local culture Collection of Tunisian Microorganisms (CTM) of the Centre of Biotechnology of Sfax (CBS Sfax-Tunisia). *Fusarium graminearum* ISPaVe 271, *Fusarium culmorum* ISPaVe 21w were provided by National School of Engineers of Sfax (ENIS Sfax-Tunisia). Fungal strains were maintained on potato dextrose agar (PDA) (Oxoid, Basingstoke, UK) medium and preserved at 4 °C. All fungal strains were conserved in 50% glycerol (PubChem CID: 753) and 50% potato dextrose broth (v/v) at −20 °C.

For spore preparation, the fungal strains were grown in PDA medium for 7 days at 25 °C. Sterile distilled water was added to the agar plate surface and spores were liberated from fungal mycelium by scraping with a sterile glass spreader. The liquid mixture was collected and filtered through Miracloth (Calbiochem, Darmstadt, Germany) before a centrifugation at 4000 rpm for 10 min. The spore pellet

was washed twice with sterile distilled water and resuspended in sterile distilled water again to obtain a final spore suspension. Fungal spore concentration was quantified under microscopy. The spore suspension obtained was adjusted to the concentration of 10^4 spores/mL. Spore suspension was directly used after preparation. Antifungal activity was determined by the agar diffusion test [28,29]. The quantity used for each extract was 50 μ L per disk (at 5 mg/mL), and the plates were incubated at 30 °C for 72 h [28]. Dimethyl Sulfoxide (DMSO) was used as a negative control, while Amphotericin B (20 μ g/wells) was used as a positive control. Antifungal activity was evaluated by measuring the diameter (mm) of circular inhibition zones around the well. Tests were performed in triplicate.

2.7.2. Determination of Minimal Inhibitory Concentration (MIC) and the Minimal Fungicidal Concentration (MFC)

For minimal inhibitory concentration (MIC) determination, each extract was dissolved in a solution of dimethylsulfoxide DMSO (PubChem CID: 679) (Loba Chemie Pvt. Ltd., Mumbai, India)/water (1/9; v/v) to a final concentration of 0.078125, 0.15625, 0.3125, 0.625, 1.25, 2.5, 5, 10 and 20 mg/mL and then filtered through 0.22 μ m pore-size black polycarbonate filters (Millipore). The test was performed in sterile 96-well microplates using 100 μ L as a final volume for each well which contain 10 μ L of cell suspension corresponding to a final concentration of 10^6 CFU/mL [30]. Positive growth control well consisted of fungi in Mueller Hinton broth (Oxoid, Basingstoke, UK) and DMSO/water (1/9) was used as negative control. The plates were then covered and incubated in appropriate temperature for 24 h. The MIC was defined as the lowest concentration in which the microorganism did not demonstrate visible growth. As an indicator of fungal growth, 25 μ L of thiazolyl blue tetrazolium bromide (MTT) (PubChem CID: 64965) (Sigma-Aldrich, Taufkirchen, Germany) was added at 0.5 mg/mL to the well microplates and kept for 30 min at 37 °C. The tetrazolium salt was a colourless electron acceptor molecule which was reduced to a red-coloured formazan product with the growth of the indicator microorganisms. This salt would remain uncoloured if the microbial growth was blocked. MIC values were determined in triplicate.

Minimal fungicidal concentrations (MFCs) were determined by serial sub cultivation of 10 μ L in PDA plates and incubated for 72 h at 28 °C. The lowest concentration with no visible growth was defined as the MFC, indicating $\geq 99.9\%$ killing of the original inoculum. The determinations of MIC and MFC values were done in triplicate. Extracts were then considered as fungistatic or fungicidal depending on the MFC/MIC ratios which were respectively greater or lesser than 4 [31].

2.7.3. Statistical Analysis

Computations were performed using the Statistical Package for the Social Sciences (SPSS) software (version 19.0; SPSS Inc., Chicago, IL, USA). The data was expressed as means $\pm$ standard deviations of three replicates. Analysis of variance was done using Tukey's post-hoc test to determine the differences among means obtained for different samples. The level of significance was set to $p < 0.05$ and Pearson correlation coefficients were generated to describe the relationship between phytochemical contents (TPC, TFC and TAC) and anti-fungal activity which was evaluated by measuring the inhibition zone (mm)

3. Results and Discussion

3.1. Physicochemical Composition

Physicochemical composition of *Spirulina* and its combinations with pomegranate peel (S1-S4) is shown in Table 1. Moisture content of ethanolic extracts was measured as a percentage of water relative to dry weight. Moisture content of pomegranate peel from 'Gabsi' cultivar used in this study (S1) was about $74.25 \pm 0.52\%$ which is slightly higher than those found in other Tunisian cultivars: 'Acide', 'Nebli' and 'Tounsi', whose contents were respectively 67.26%; 72.58% and 72.68% [32]. As illustrated in Table 1, moisture content of sample S2 (*Spirulina*) was $8.25 \pm 0.07\%$. This value was higher

than *Hawaiien Spirulina* Pacifica (4.70%) and Algerian *Spirulina* (5.17%) but lower than the *Spirulina* moisture content from Chad (8.40%) [33]. In this study, moisture content was less than 10%, which is the recommended condition for long-term storage of *Spirulina* powders. On the other hand, the combination S4 showed a significant difference ($p < 0.05$) compared to S2 for moisture content values.

Table 1. Physicochemical composition (moisture (%), dry matter (%), protein (%), lipid (%) and ash (%)) and phytochemical content total polyphenols content: TPC (mg GAE/g), total flavonoids content: TFC (mg QE/g) and total anthocyanins content: TAC (mg cy-3-glu/100 g) of the extracts from *Spirulina platensis* and its combinations with pomegranate peel.

		S1	S2	S3	S4
Chemical Properties	Moisture	74.25 ± 0.52 ^c	8.25 ± 0.07 ^a	11.85 ± 0.13 ^a	41.35 ± 0.47 ^b
	Dry matter	97.51 ± 4.55 ^c	94.75 ± 3.77 ^b	93.63 ± 3.33 ^{ab}	92.65 ± 2.52 ^a
	Protein	7.68 ± 0.22 ^a	69.54 ± 3.44 ^d	54.52 ± 3.01 ^c	37.42 ± 2.68 ^b
	Lipid	Nd	7.18 ± 0.42 ^c	5.15 ± 0.28 ^b	2.75 ± 0.19 ^a
	Ash	5.02 ± 0.51 ^a	10.69 ± 0.32 ^c	8.07 ± 0.49 ^b	7.54 ± 0.23 ^b
Phytochemical Content	TPC	131.14 ± 5.25 ^d	4.59 ± 0.21 ^a	34.5 ± 1.72 ^b	66.75 ± 3.25 ^c
	TFC	6.75 ± 0.25 ^d	2.75 ± 0.05 ^a	3.41 ± 0.12 ^c	4.27 ± 0.18 ^b
	TAC	24.51 ± 1.15 ^d	0.51 ± 0.01 ^a	5.87 ± 0.05 ^b	12.02 ± 0.65 ^c

S1 (pomegranate peel), S2 (*Spirulina*), S3 (pomegranate peel—*Spirulina*: 25/75%) and S4 (pomegranate peel—*Spirulina*: 50/50%). ±: standard deviation of the mean; values with a different letter (a–d) of the same chemical properties or phytochemical content are significantly different ($p < 0.05$).

The protein content of the studied samples varied significantly ($p < 0.05$) between 7.68 and 69.54% (Table 1). *Spirulina* (S2) has the highest ($p < 0.05$) protein content (69.54%) compared to S1, S3 and S4. These contents are also higher than those found in other *Spirulina* strains [34,35]. Amongst others proteins, phycocyanin is a blue coloring agent applied in the food industry and cosmetics, stands out with a well-established antioxidant capacity [36].

For S1 sample, protein content was higher than those found in other Tunisian cultivars ‘Acide’ (3.96%), ‘Nebli’ (5.42%) and ‘Tounsi’ (5.84%) [32], as well as other Turkish cultivars, ‘Lefon’, ‘Seedless’, ‘Kadi’, ‘Siyah’ and ‘Koycegiz’, whose contents are 3.19; 3.11; 3.06; 2.67 and 2.58%, respectively [37]. The protein content in S3 sample (54.52%) was found to be higher ($p < 0.05$) than the other combined sample (S4: 37.42%).

As presented in the Table 1, S2 sample showed the highest ($p < 0.05$) total lipid content (TLC) of 7.18%, for the other samples TLC values were about 0, 5.15 and 2.75 for S1, S3 and S4, respectively. In accordance with our results, Bensehaila et al. [35] found a similar TLC of 7.28% for a *Spirulina platensis* strain. However, in the same condition of growth, Algerian *Spirulina* showed lower lipid content [33]. According to Capelli and Cysewski [38], the lipid content in *Spirulina* ranged between 5 and 8%, but as reported by Babadzhanov et al. [39], it could reach 14.3% in an Uzbekistan *Spirulina platensis* strain. This variability could be related to the differences in culture conditions. In the same context, Richmond [40] reported that microalgae lipid production depends on the species and their culture conditions such as nutrients, salinity, light intensity, temperature, pH and even the association with other microorganisms.

The total ash content of studied samples was between 5.02% and 10.69%, for S1 and S2 samples respectively. The concentration of ash found in pomegranate peels (S1) was higher than that reported by Abid et al. [32], ranging between 3.71% and 4.97%, and lower than the values presented by Romelle et al. [41]. As shown in the Table 1, *Spirulina* samples (S2) showed ash percentage of 10.69%; this value was comparable to *A. platensis* from Algeria and higher compared with previous work by Bensehaila et al. [35].

3.2. Phytochemical Content

Concerning the phytochemical content, comparison between the four samples showed that TPC in S1 was significantly higher ($p < 0.05$) than those in S2, S3 and S4. Levels of TPCs ranged between 4.59 and 131.14 mg GAE/g of dried sample (Table 1). The best TPC (131.14 mg GAE/g of extract) was obtained from the S1 sample, consistent with previous studies [32], the TPCs of four ethanolic extracts from different Tunisian ecotypes ranged between 109.21 and 140.93 mg GAE/g. For S2 samples, results indicated the presence of TPC at a concentration of 4.59 mg GAE/g. This value was higher than those found in other studies reported by De Marco et al. [42], Gargouri et al. [43] where the authors demonstrated that the TPCs were, respectively, 4.08, 3.4 and 2.49 mg GAE/g. As shown in Table 1, the determination of major classes of secondary compounds in S1, S3 and S4 extracts indicated that phenolic compounds were high in anthocyanins at 18.7%, 17.01% and 18.00%, respectively, while *Spirulina* (S2) was rich in flavonoids (59.91%). Gargouri et al. [43] reported that *Spirulina* has high amount of flavonoids with TFC/TPC ratios of 35.29% and 76.30%, respectively.

3.3. Antifungal Activity against Plant Pathogenic Fungi

Various pathogenic fungi infect plants leading to the reduction in yield and quality crop, and the contamination of grains with fungal mycotoxins. Therefore, we have evaluated the antifungal activities of S1, S2, S3 and S4 against five fungal plant pathogens namely *F. oxysporum*, *F. culmorum*, *F. graminearum*, *Alternaria alternata* and *Aspergillus niger*.

As shown in Table 2, the antifungal test demonstrated that pomegranate peel (S1) produced antifungal compounds against a variety of phytopathogenic fungi from different classes. A moderate but significant ($p < 0.05$) inhibitory effect of S1 on the growth of *Aspergillus niger* CTM 10099 with an inhibition zone of 14 mm was observed. In 2009, Al-Zoreky [44] reported that several fungal species, such as *Aspergillus niger*, were sensitive to pomegranate fruit peel water/methanol extract, however, the inhibitory effect of pomegranate water extracts have not been studied against this fungi. In earlier studies, Azzouz and Bullerman [45] in 1982 reported that pomegranate peel extract have no effect on the growth of *Aspergillus flavus* and *Aspergillus parasiticus*. More recently, Rongai et al. [46] reported in 2017 that aqueous extract of pomegranate peel showed a wide spectrum of antifungal activities especially against *Fusarium oxysporum*.

Table 2. Antifungal activity of of *Spirulina platensis* and its combinations with pomegranate peel against plant pathogenic fungi: *Fusarium oxysporum*, *Fusarium culmorum*, *Fusarium graminearum*, *Aspergillus niger* and *Alternaria alternata* strains.

Fungal Strains	Inhibition Zones Diameters (mm)				
	S1	S2	S3	S4	Amphotericin B
<i>Fusarium oxysporum</i> (CTM10402)	12.25 ± 0.25 aB	15.75 ± 0.75 abB	22.25 ± 0.50 dB	16.25 ± 0.50 cC	14.25 ± 0.25 bB
<i>Fusarium culmorum</i> (ISPAVE 21w)	10.00 ± 0.75 aA	15.00 ± 0.33 bB	21.75 ± 0.33 dB	15.00 ± 0.33 cB	12.50 ± 0.75 bA
<i>Fusarium graminearum</i> (ISPAVE 271)	11.75 ± 0.33 aAB	15.25 ± 0.38 bB	24.75 ± 0.33 dC	12.75 ± 0.50 bA	14.75 ± 0.50 c
<i>Aspergillus niger</i> (CTM 10099)	14.00 ± 0.83 aC	11.25 ± 0.66 bC	18.25 ± 0.75 cA	14.75 ± 0.38 abB	15.25 ± 0.83 bC
<i>Alternaria alternata</i> (CTM 10230)	11.75 ± 0.66 aAB	10.25 ± 0.25 aA	19.25 ± 0.83 cA	14.25 ± 0.66 bB	14.75 ± 0.66 bBC

S1 (pomegranate peel), S2 (*Spirulina*), S3 (pomegranate peel—*Spirulina*: 25%/75%) and S4 (pomegranate peel—*Spirulina*: 50%/50%). ±: standard deviation of the mean; inhibition zones diameters (mm) with a different letter (a–d) of the same fungal strain are significantly different ($p < 0.05$); Inhibition zones diameters (mm) with a different letter (A–C) of the same extract are significantly different.

Equally, as illustrated in the Table 2, we have demonstrated the capacity of *Spirulina* (S2) to inhibit all the studied panel of fungal strains, particularly *Fusarium* genus. In fact, this antifungal activity was similar to standard synthetic antifungal agent (Amphotericin B). Similarly, Al-Ghanayem [47], reported the inhibition growth of *Fusarium oxysporum* followed by *Aspergillus flavus* and *Aspergillus niger* by *Spirulina platensis* organic extract. Furthermore, Kumar et al. in 2011 reported the inhibitory effect of hexane and methanolic extracts of *Spirulina platensis* against *Aspergillus* spp. [48]. The activity of the alga could be due to the intracellular and extracellular metabolites that have antifungal properties [47]. In general, it was demonstrated that the most antifungal compounds in *Spirulina* are mainly polyphenols along with polysaccharides that inhibit microbial growth, or directly by destroying the living structures of fungi [49]. Compared to S1, S2 and S4, Amphotericin B had higher antifungal activity against *Aspergillus niger* CTM 10099 and *Alternaria alternata* CTM 10230. Concerning anti *Fusarium* activity, *Spirulina* (S1) had a significantly ($p < 0.05$) lower inhibition zone compared to Amphotericin B (Table 2).

Interestingly, the extract S3 revealed a strong ($p < 0.05$) antifungal activity against all tested species, and the distance of the inhibitory zone varied from 18.25–24.75 mm (Table 2). The *Fusarium graminearum* ISPAVE 271, was the most susceptible with an inhibition zone of 24.75 mm followed by *Fusarium oxysporum* CTM10402, *Fusarium culmorum* ISPAVE 21w, *Alternaria alternata* CTM 10230 and *Aspergillus niger* CTM 10099. S3 showed a strong activity against mycotoxigenic and phytopathogenic fungi, notably against *F. graminearum*, *F. oxysporum* and *F. culmorum* producing type B trichothecene mycotoxins, nivalenol (NIV), deoxynivalenol (DON) and their acetylated derivatives. However, moderate activity was found against *Aspergillus niger* the main ochratoxin A (OTA) producer (Table 2).

3.4. MIC and MFC Determination

The quantitative evaluation of the antifungal activity of S1, S2, S3 and S4 extracts was carried out. The effect of each extracts on the growth of all fungal strains was thus tested at different concentrations (0.078–20 mg/mL). The MIC and the MFC were determined and ratio of MFC/MIC was obtained (Table 3). The observed MIC values of the S1 (pomegranate peel) extract showed same values for the three strains: *Fusarium culmorum* ISPAVE 21w, *Fusarium graminearum* ISPAVE 271 and *Alternaria alternata* CTM 10230 strains (0.312 mg/mL), while S2 (*Spirulina*) extract MIC value was about 0.156 mg/mL for all three strains. Some differences could be observed with previously reported literature. For example, Usharani et al. in 2015 stated that the ethanolic *Spirulina* extracts exhibited antifungal activity against *A. niger* at concentrations of 16, 35 and >35 mg/mL [50]. The MFC/MIC ratio was also calculated and an agent was considered fungicidal if the minimal fungicidal concentration (MFC) to minimal inhibitory concentration (MIC) ratio was ≤ 4 and fungistatic if the ratio was >4 . MFC/MIC ratio indicated that S3 and S4 extracts showed interesting fungicidal effects on all studied pathogens fungi (Table 3). Comparing the mean of MIC and MFC values of all tested fungi strains, S3 and S4 exhibited approximately two times stronger inhibition than S1 and S2. Remarkably, S3 and S4 extracts exhibited the lowest ratio MFC/MIC values against all fungi with a value equal to 2 (Table 3). The combination of *Spirulina* and pomegranate peel extracts showed a synergistic antifungal effect against all fungal strains. According to these observations, strong antifungal activity could be attributed to phenolic compounds (Table 1). Possible modes of action of phenolic compounds have been reported in different reviews [51–54], however the mechanisms have not been completely elucidated. López-Malo et al. in 2005 mentioned that the effect of phenolic compounds is concentration-dependent [51]. The authors reported that, at low concentration, phenols could affect enzyme activity negatively, especially of those associated with energy production, while at greater concentrations, protein denaturation could occur [51]. The effect of phenolic compounds on fungal growth and toxin production could be the result of cell permeability alteration, permitting the loss of macromolecules from the interior. They could also interact with membrane proteins, causing a deformation in their structure and functionality [51]. Once the phenolic compound crossed the cellular membrane, interactions with membrane enzymes and proteins would cause an opposite flow of protons, affecting cellular activity [51–54].

Table 3. Minimal inhibitory concentration (MIC) and minimal fungicidal concentration (MFC) values of *Spirulina platensis* and its combinations with pomegranate peel against *Fusarium oxysporum*, *Fusarium culmorum*, *Fusarium graminearum*, *Aspergillus niger* and *Alternaria alternata* strains.

	Extract	MIC	MFC	Ratio MFC/MIC	Interpretation
<i>Fusarium oxysporum</i> (CTM10402)	S1	0.312	1.250	4	Fungicidal
	S2	0.156	0.625	4	Fungicidal
	S3	0.078	0.156	2	Fungicidal
	S3	0.156	0.312	2	Fungicidal
<i>Fusarium culmorum</i> ISPAVE 21w	S1	0.312	1.250	4	Fungicidal
	S2	0.156	0.625	4	Fungicidal
	S3	0.078	0.156	2	Fungicidal
	S4	0.078	0.156	2	Fungicidal
<i>Fusarium graminearum</i> ISPAVE 271	S1	0.312	1.250	4	Fungicidal
	S2	0.156	1.250	8	Fungistatic
	S3	0.078	0.156	2	Fungicidal
	S4	0.078	0.156	2	Fungicidal
<i>Aspergillus niger</i> CTM 10099	S1	1.250	5	4	Fungicidal
	S2	0.625	2.5	4	Fungicidal
	S3	0.312	0.625	2	Fungicidal
	S4	0.312	0.625	2	Fungicidal
<i>Alternaria alternata</i> CTM 10230	S1	0.312	1.250	4	Fungicidal
	S2	0.156	0.312	2	Fungicidal
	S3	0.078	0.156	2	Fungicidal
	S4	0.156	0.312	2	Fungicidal

S1 (pomegranate peel), S2 (*Spirulina*), S3 (pomegranate peel—*Spirulina*: 25%/75%) and S4 (pomegranate peel—*Spirulina*: 50%/50%).

3.5. Relationships between Phytochemical Content of Pomegranate Peel, *Spirulina* and Their Combinations and Antifungal Activities

The correlation between phytochemical content (TPC, TFC and TAC) and antimycotoxigenic fungal activities was investigated. Therefore, as shown in Table 4, for S1 samples, a significant negative correlation was found between TPC, TAC and five mycotoxigenic fungi. This negative correlation was more significant for TAC in anti *F. oxysporum* CTM10402 ($r = -0.708$, $p < 0.05$), anti *F. culmorum* ISPAVE 21w ($r = -0.861$, $p < 0.01$) and *A. niger* CTM 10099 ($r = -0.837$, $p < 0.05$) samples. These data consolidate our results shown in Table 1, in which the phenolic compounds (TFC) were rich in anthocyanins (TAC) for S1 samples. Our results are in agreement with those previously reported by Glazer et al. [55] where a correlations between TFC and the growth rates of *A. alternata* ($r = -0.85$, $p < 0.01$) and *Fusarium* spp., ($r = -0.89$, $p < 0.05$) was found. It is known that TPC, TAC and antibacterial activity are usually well correlated in red color-anthocyanin rich fruits [56]. Furthermore, contrary to the study of Duman et al. [57], no significant correlation ($p > 0.05$) was found between TPC, TAC and the yeast *Candida albicans*. As presented in Table 4, a significant positive correlation was found between TPC, TFC and all fungi strains. In fact, for S2 samples, the TFC content showed a strong correlation ($p < 0.01$) with the anti *F. graminearum* ISPAVE 271 ($r = 0.983$), anti *A. alternata* CTM 10230 ($r = 0.983$) and anti *A. niger* CTM 10099 ($r = 0.878$). In our case, the use of raw phenolic extract from *Spirulina* sp. and pomegranate peel in combination (S3 and S4) exhibited promising growth inhibitory profile against fungal strains. The phenolic group joined to a single hydroxyl group confers lipophilicities and acidity, important factors in antifungal activity [58]. Phenolic compounds primarily synthesized through the pentose phosphate pathway (PPP), shikimate and phenylpropanoid pathways. The oxidative PPP provides erythrose-4-phosphate precursor for the shikimate pathway which converts these sugar phosphates to aromatic amino acids like phenylalanine, which becomes the precursor for the phenylpropanoid pathway. This pathway is responsible for the synthesis of a wide variety of phenolic compounds [59] and plays a vital role in plant growth, regulation of plant metabolism,

lignin synthesis and exhibit pharmacological properties such as antitumor, antiviral, antiinflammatory, hypotensive and antifungal activity [59,60].

Table 4. Pearson's correlation coefficients between the antimicrobial (antibacterial and antifungal) activity and the phytochemical contents.

Fungi Strains		TPC	TFC	TAC
<i>F. oxysporum</i> CTM10402	S1	−0.700 *	0.006	−0.708 *
	S2	0.673 *	0.687 *	−0.172
	S3	0.878 **	0.408	−0.675 *
	S4	0.723 *	0.088	−0.622 *
<i>F. culmorum</i> ISPAVE 21w	S1	−0.774 *	0.640	−0.861 **
	S2	0.763 *	0.641 *	−0.259
	S3	0.878 **	0.408	−0.675 *
	S4	0.956 **	0.457	−0.664 *
<i>F. graminearum</i> ISPAVE 271	S1	−0.809 *	0.268	−0.756 *
	S2	0.652 *	0.983 **	−0.335
	S3	0.994 **	0.809 *	−0.747 *
	S4	0.681 *	0.760 *	−0.157
<i>A. niger</i> CTM 10099	S1	−0.732 *	0.595	−0.837 *
	S2	0.738 *	0.878 **	−0.404
	S3	0.700 *	0.981 **	−0.743 *
	S4	0.670 *	0.726 *	−0.664 *
<i>A. alternata</i> CTM 10230	S1	−0.758 *	0.311	−0.700 *
	S2	0.752 *	0.983 **	−0.335
	S3	0.923 **	0.758 *	−0.284
	S4	0.904 **	0.657 *	−0.534

*: $p < 0.05$; **: $p < 0.01$; Notes: The most significant loadings are highlighted in bold.

For S3 and S4 samples, a significant correlation was found between: (*F. graminearum* ISPAVE 271, TPC, TFC and TAC) and (*A. niger* CTM 10099 and all phytochemical contents). For S3 samples, *F. oxysporum* CTM10402 and *F. culmorum* ISPAVE 21w were only correlated with TPC and TAC (Table 4), while *A. alternata* CTM 10230 was correlated with TFC ($r = -0.758$, $p < 0.05$). Interestingly, for the S4 sample, anti *A. niger* CTM 10099 activity had a high correlation with TPC ($r = 0.670$, $p < 0.05$); TFC ($r = 0.726$, $p < 0.05$) and TAC ($r = -0.664$, $p < 0.05$). Equally, as shown in the Table 4, a strong relationship was found between anti *A. alternata* CTM 10230 activity, TPC ($r = 0.904$, $p < 0.01$) and TFC ($r = 0.657$, $p < 0.05$). Thus, the synergistic effects, and the diversity of TPC, TFC and TAC presented in the combination of pomegranate peel and *spirulina* ethanolic extracts should be taken into account for their antifungal activities.

4. Conclusions

Spirulina and pomegranate peel extracts alone and in combination were chemically characterized and then evaluated for their antifungal activities against *Fusarium*, *Aspergillus* and *Alternaria* genera. Correlations between these antifungal activities and phytochemical content were evaluated. The results showed that the combination between *Spirulina* at 75% and pomegranate peel at 25% (S3) have a significant impact ($p < 0.05$) on the antifungal activity compared to S1, S2 and S4. Finally, Pearson correlation demonstrated that S3 polyphenols were mostly responsible for tested antimycotoxigenic and antiphytopathogenic fungal properties. Therefore, bioactive molecules of *Spirulina* and pomegranate peel combination could be used as a safe method against plant pathogenic fungi. To the best of our knowledge, this is the first work reporting the combination of algae and a plant by-product to enhance the antifungal activity.

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