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Phenolic Content, Antioxidant and Antimicrobial Properties of Hawthorn (*Crataegus orientalis*) Fruit Extracts Obtained via Carbohydrase-Assisted Extraction

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Abstract: The enzyme-assisted approaches for plant phenolics extraction are more eco-friendly methods compared to acid or alkaline hydrolysis. Carbohydrase enzymes can release free phenolics from plant materials by cleaving the glycosidic bonds between phenolic compounds and cell wall polymers. In this study, the efficiency of carbohydrase-assisted treatment approaches was evaluated to extract bioactive phenolics from hawthorn (*Crataegus orientalis*) fruit residues. Enzymatic treatment of the fruits was operated by using a crude cellulolytic enzyme cocktail from *Rhizomucor miehei* NRRL 5282 and a pectinase preparate from *Aspergillus niger*. Both cellulase and combined cellulase–pectinase treatments improved the total phenolic content (TPC) and antioxidant activity of extracts. The TPC increased to 1899 ± 27 mg gallic acid equivalents/100 g dry matter during the combined enzyme treatment, showing a strong correlation with the average antioxidant capacity determined by ferric-reducing antioxidant power (1.7-fold increment) and 2,2-diphenyl-1-picrylhydrazyl (1.15-fold increment) reagents. The major phenolics in enzyme-treated extracts were vanillic and ferulic acids, the concentrations of which increased 115.6-fold and 93.9-fold, respectively, during carbohydrase treatment. The planktonic growth of *Escherichia coli*, *Bacillus subtilis*, *Pseudomonas aeruginosa*, and *Chromobacterium violaceum* was slightly inhibited by the extracts with minimum inhibitory concentration values between 15.0 and 77.9 mg/mL, while the yeasts tested were quite resistant to the samples. *B. subtilis* and yeast biofilms were sensitive to the enzyme-treated extracts, which also showed quorum-sensing inhibitory effects against *C. violaceum*. The obtained bioactive hawthorn extracts hold potential as a natural source of antioxidants and antimicrobials.

Keywords: hawthorn; enzyme-assisted extraction; phenolic antioxidants; cellulase; pectinase; antimicrobial activity; biofilm inhibition; quorum-sensing inhibition

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1. Introduction

Crataegus species (hawthorn) are widely distributed in Europe, Asia, North Africa, and North America, and the leaf, flower, and fruit parts of the plant have been used in folk medicine since ancient times [1,2]. In herbal teas, the flowering branch tips are used, but hawthorn fruits are also appreciated. In some countries, the fruits are consumed raw or

processed into jam or beverages [3,4]. Hawthorn fruits are rich sources of polyphenols, flavonoids, vitamins, organic acids, tannins, and other phytochemicals [5,6]. Hawthorn fruit extracts exhibit several pharmacological activities, including anti-inflammatory, cardioprotective, gastroprotective, antimicrobial, immunostimulant, hypoglycaemic, and cytotoxic properties [2].

Bioactive phenolics can possess health-protective effects, including antioxidant, antimicrobial, anti-inflammatory, and anti-cancer activities; they can be found in large amounts in cultivated or wild fruits [7]. Some phenolic compounds in hawthorn have shown significant inhibitory effects against α-glucosidase, making them promising anti-diabetic agents [8]. Many of these phenolic compounds can be used in the food industry as additives in functional foods or as preservatives in packaged foods [9–13]. For instance, phenolic extracts were applied in yogurt [14], bread [15], fresh pasta [16], and cottage cheese [17] products and tested as a natural antioxidant additive. However, the majority of plant phenolics can be found in phenolic–saccharide complexes, mainly in ester and glycosidic linkages, in the plant cell wall, resulting in limited bioavailability for the phenolic compound.

Free phenolic substances can be obtained in high amounts from their bounded form through a variety of extraction methods. Widely used chemical methods for the release of phenolics are the acid and alkaline hydrolysis methods; however, these treatments often result in increased yields of harmful byproducts as well as low-quality final products [18,19]. Effective phenolic extraction techniques also include ultrasonic- and microwave-assisted treatments, as well as supercritical fluid extraction methods [20]. Green technology for antioxidant compound extraction is a subcritical water extraction method [21]. A non-thermal, nontoxic, specific, and eco-friendly approach is the enzyme-assisted technique for the substrate treatment [19,22,23]. For instance, the carbohydrate-cleaving enzymes, including cellobiohydrolases (EC 3.2.1.91) and β-glucosidases (EC 3.2.1.21), can hydrolyze the glycosidic bond between the phenolic compound and the sugar residue, releasing the phenolic aglycones [24]. Pectinases (EC 3.2.1.15) can decrease the tissue rigidity in plant cell walls, also supporting the phenolic release from the polyphenolic–saccharide structures [25]. Although hawthorn fruit has a considerable number of phenolic glycosides, extracting them via enzyme-assisted approaches has not yet been investigated.

In a previous study, the combination of a *Rhizomucor miehei* cellulolytic enzyme cocktail produced on wheat bran and a commercial *Aspergillus niger* pectinase was used for the successful extraction of antioxidative phenolic compounds with antimicrobial properties from black grape, apple, and pitahaya residues [26,27]. Here, an attempt was made to apply the *R. miehei* cellulase and *A. niger* pectinase-containing enzyme mixtures to obtain free phenolic compounds from hawthorn fruits. Individual phenolic compound profiles as well as the antioxidant and antimicrobial capacities of the extracts were also examined.

2. Materials and Methods

2.1. Plant Material

Hawthorn fruits (*Crataegus orientalis* Pall. ex M.Bieb. subsp. *orientalis*, Figure 1) were collected in the area of Takkalı Mountain, Konya, Turkey, at an altitude of 1520 m. The plant sample was identified by Osman Tugay. A voucher specimen was stored at KNYA Herbarium, Selçuk University, Turkey (accession number 15306). After collection, the fruits were stored in a refrigerator (8 °C) until preparation for extraction.

2.2. Microorganisms and Growth Conditions

The filamentous fungus *R. miehei* NRRL 5282 (SZMC 11005), along with bacterial strains such as *Escherichia coli* SZMC 0582, *Pseudomonas aeruginosa* SZMC 0568, *Bacillus subtilis* SZMC 0209, and *Chromobacterium violaceum* 85WT (SZMC 6269), and yeast strains such as *Wickerhamomyces anomalus* (formerly known as *Pichia anomala*) SZMC 8061Mo, and *Debaryomyces hansenii* SZMC 8045Mo, were sourced from the Szeged Microbiology Collection (SZMC, Szeged, Hungary; http://szmc.hu/, accessed on 4 September 2024).

Figure 1. Fruit details of *Crateagus orientalis* Pall. ex M.Bieb. subsp. *orientalis*. Photo by O. Tugay.

The *E. coli*, *P. aeruginosa*, and *C. violaceum* were grown on lysogeny broth (Luria-Bertani medium, LB; tryptone, 10 g/L; yeast extract, 5 g/L; NaCl, 5 g/L) at 37, 30 or 25 °C, respectively. The *B. subtilis* was grown on tryptone, glucose, and yeast extract medium (TGE; glucose, 1 g/L; yeast extract, 3 g/L; tryptone, 5 g/L) at 30 °C. The *R. miehei*, *W. anomalus*, and *D. hansenii* were grown on malt extract medium containing 5 g/L of yeast extract, 5 g/L of glucose, and 50 mL/L of 20% (*v*/*v*) malt extract at 37 °C (for *R. miehei*) or 30 °C (for *W. anomalus* and *D. hansenii*). All ingredients were purchased from VWR International (Radnor, PA, USA). Fresh fungal and bacterial cultures were prepared under the corresponding growth conditions with a 24 h incubation before each experiment. The growth of each bacterium and yeast used for antimicrobial tests was in the stationary phase at the end of the incubation. After a dilution step in the respective growth medium, the cell number was determined in a Bürker chamber under a light microscope.

2.3. Enzyme Cocktail Preparation

The crude cellulolytic enzyme cocktail was obtained in pH 5.0 acetate buffer by solid-state fermentation of *R. miehei* on wheat bran as described in detail by Takó et al. [28]. The crude cellulolytic enzyme cocktail contained 44.5 U of filter paper degrading, 121.5 U of endoglucanase, 21.4 U of cellobiohydrolase, and 2149.3 U of β-glucosidase activities. A commercially available *A. niger* pectinase (Sigma-Aldrich, Munich, Germany) with 1.1 U/mg polygalacturonase activity was used in the combined enzyme treatments.

2.4. Preparation of Fruit Material and Enzyme-Assisted Extraction

The production steps of the hawthorn extracts are summarized in Figure 2. Whole fruits were dried at 65 °C to constant weight (18–20 h). The dried material was ground to a fine powder and stored at room temperature until use. For enzyme-assisted extraction, the crude *R. miehei* cellulolytic enzyme extract, alone (designated as S1 sample) or in combination (designated as S2 sample) with *A. niger* pectinase (5 mg), was added to 5 g of fruit powder in 50 mL of acetate buffer (50 mmol/L, pH 5.0). The control sample was extracted with acetate buffer (50 mmol/L) alone without any enzyme (designated as C sample). Both enzyme-assisted and enzyme-free extractions of the hawthorn fruits were maintained for 5 h at 50 °C in an orbital shaker (KS 4000 ic control, IKA, Staufen, Germany) at 200 rpm. After that, the reaction was stopped by boiling the mixture for 3 min, and the mixtures were centrifuged (Sorvall RC 6 Plus, Thermo Fisher Scientific, Waltham, MA, USA) at 16,200× *g* for 10 min. The supernatant was then filtered through a 20 µm filter (Millex-GN, nylon, Merck Millipore Ltd., Carrigtwohill, Ireland) and sterilized using a 0.45 µm filter (Millex-HV, PVDF, Merck Millipore Ltd., Carrigtwohill, Ireland). The sterilized extract was stored at −20 °C until use. Extract concentrations were 120.25, 176.25, and 155.75 mg/mL for the C, S1, and S2 samples, respectively, determined by lyophilizing (Christ Alpha 1–4 freeze dryer, Martin Christ GmbH, Osterode am Harz, Germany) a 1 mL extract sample until completely dry and then measuring the weight of the remaining dry

material. For antimicrobial investigations, stock solutions with concentrations of 240.5, 352.5, and 311.5 mg/mL were prepared from C, S1, and S2 samples, respectively, in 10% (v/v) ethanol.

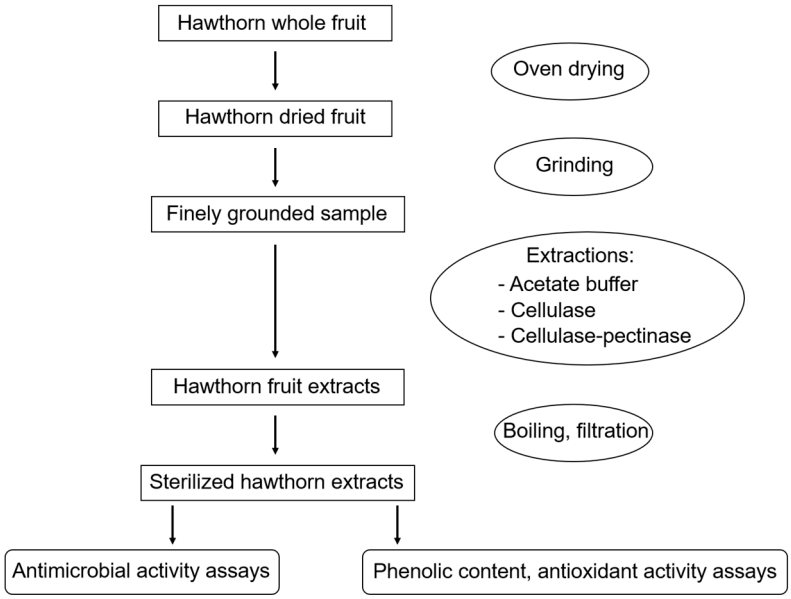

Figure 2. Flowchart diagram of the production process of hawthorn extracts.

2.5. Total Phenolic Content Assay

The total phenolic content (TPC) was determined using the Folin–Ciocalteu assay in microtiter plates. A volume of 20 µL of hawthorn extract diluted to 10-fold in ethanol (96%) was mixed with 10 µL of 50% Folin–Ciocalteu's phenol reagent (Sigma-Aldrich, Munich, Germany), 20 µL of ethanol (96%), and 100 µL of distilled water. After 5 min of incubation at room temperature, 20 µL of sodium carbonate (5%) was added to initiate the reaction. The reaction mixture was incubated for 60 min in dark conditions, then absorbance was measured at 725 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany). Gallic acid in the concentration range of 0–100 µg/mL was used to establish the standard curve. TPC was expressed as mg of gallic acid equivalents (GAE) in 100 g dry matter (DM).

2.6. Determination of Antioxidant Activity

The antioxidant activity was determined by measuring the ferric reducing antioxidant power (FRAP) and the free radical scavenging activity of the enzyme extracts in microdilution tests.

For FRAP analysis, first, a reagent solution was prepared containing 80 mL of 300 mM acetate buffer (pH 3.6), 8 mL of 10 mM 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ; Sigma-Aldrich, Munich, Germany) diluted in 40 mM HCl, 8 mL of 20 mM iron(III) chloride, and 4.8 mL of distilled water. Then, 6 µL of extract diluted in 50% (v/v) ethanol (10-fold dilution) was transferred to 200 µL FRAP reagent. After vortexing, the reaction mixture was incubated at 37 °C for 30 min, and the absorbance was determined at 593 nm (SPECTROstar Nano, BMG Labtech, Offenburg, Germany). The calibration curve was established using a 1 mM iron(II) sulfate solution in the concentration range of 0.1–1.0 mM. The FRAP of the hawthorn extracts was expressed as µM Fe(II)/g DM.

The free radical scavenging capacity was determined using the 2,2-diphenyl-1-picrylhydrazyl (DPPH; Sigma-Aldrich, Munich, Germany) reagent. For the reaction, 150 µL of 100 µM DPPH solution prepared freshly in 80% (v/v) ethanol was mixed with 25 µL of extract diluted in 50% (v/v) ethanol (10-fold dilution). The control sample contained 150 µL of 100 µM DPPH and 25 µL of 50% (v/v) ethanol. After a 25-min incubation of

both the reaction mixture and the control sample in a dark place at room temperature, the absorbance was measured at a wavelength of 517 nm (SPECTROstar Nano, BMG Labtech, Offenburg, Germany). The results were plotted as percentage DPPH radical scavenging capacity against the control. The following equation was used to calculate the free radical scavenging activity:

$$\text{DPPH (\%)} = [(A_{control} - A_{extract})/A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the control sample, and $A_{extract}$ is the absorbance of the reaction mixture containing the diluted hawthorn extract.

2.7. Liquid Chromatography–High Resolution Mass Spectrometry (LC-HRMS)

LC-HRMS measurements were performed using a Dionex Ultimate 3000 UHPLC system (Thermo Fisher Scientific, Waltham, MA, USA) coupled to a Q Exactive Plus hybrid quadrupole-Orbitrap mass spectrometer operating with a heated electrospray interface (HESI). The phenolic compounds were separated using a Gemini-NX C18 (3 µm, 150 × 2 mm) column (Phenomenex, Torrance, CA, USA). Water/methanol (9/1) (A) and methanol (B), both supplemented with 0.1% acetic acid, served as mobile phases. The linear gradient started with 5% B and was held for 1 min. After increasing to 95% B in 11 min, it was maintained for 5 min. After reaching the initial solvent composition in 1 min, it was held for 6 min for re-equilibration. The flow rate was 0.2 mL/min, the injection volume was 5 µL, and the column temperature was maintained at 25 °C. All samples were analyzed in negative ionization mode. The ion source had the following settings: probe heater temperature, ion transfer capillary temperature, spray voltage, sheath gas flow rate, auxiliary gas flow rate, and S-lens RF level were set to 300 °C, 350 °C, 3.5 kV, 30 arbitrary units, 10 arbitrary units, and 50 arbitrary units, respectively. The mass spectrometer acquired data using a full-scan/data-dependent MS/MS method (Full MS/ddMS2). The full-scan MS spectra were acquired at a resolution of 70,000 from m/z 100 to 600 with a maximum injection time of 100 ms. For every full scan, 10 ddMS2-scans were performed with a resolution of 17,500 and a minimum automatic gain control target of 2.00×10^5. The isolation window was 0.4 m/z. LC-HRMS data were acquired using Trace Finder 4.0 software (Thermo Fisher Scientific, Waltham, MA, USA). The raw MS data files were processed using Compound Discoverer™ (2.1) software (Thermo Fisher Scientific, Waltham, MA, USA).

2.8. Determination of Minimum Inhibitory Concentrations (MICs)

The MICs of the hawthorn extracts obtained by acetate buffer and enzyme-assisted extractions on the growth of selected bacteria and yeast were determined by a microdilution assay [27]. First, the stock solutions of each hawthorn extract were serially diluted with 10% (v/v) ethanol at concentrations of 0.93 to 120.25 mg/mL, 1.37 to 176.25 mg/mL, and 1.21 to 155.75 mg/mL for the C, S1, and S2 samples, respectively. A volume of 100 µL from the diluted samples and the corresponding stock solutions were added to each well of a 96-well sterile microtiter plate (Sarstedt, Nümbrecht, Germany). Then, a volume of 100 µL of bacterial or yeast cell suspension (10^6 CFU/mL) prepared in double concentrated growth medium was added to each well. The content of the C, S1, and S2 samples in the wells thus depicted a range of 0.46 to 120.25 mg/mL, 0.68 to 176.25 mg/mL, and 0.61 to 155.75 mg/mL, respectively. Positive controls contained 100 µL of inoculated growth medium and 100 µL of 10% (v/v) ethanol solution, while the mixtures of 100 µL of diluted hawthorn extracts and 100 µL of double-concentrated medium were considered as negative controls. Absorbance measurements were made for each plate at 620 nm (SPECTROstar Nano spectrophotometer, BMG Labtech, Offenburg, Germany) before incubation. The plates were then incubated for 24 h under appropriate cultivation conditions and the absorbance was measured again at the end of the incubation. The lowest concentration of the corresponding hawthorn extract that caused 90% or higher growth inhibition was considered as the MIC.

2.9. Determination of the Anti-Biofilm Activity of the Extracts

The crystal violet assay [29] was applied to evaluate the biofilm-forming inhibitory potential of hawthorn extracts. A volume of 200 µL of 24 h old bacterial or yeast culture (approximately 10^8 CFU/mL) was transferred to the wells of a 96-well polystyrene microtiter plate (Sarstedt, Nümbrecht, Germany). The plates were then incubated for 4 h at temperatures appropriate for the growth of bacteria or yeast tested. Subsequently, the non-adherent cells were removed, and the plates were rinsed with physiological saline. After 10 min of drying, 200 µL of fresh medium containing hawthorn extracts was added to each treated well. For bacteria, the MIC/2 extract concentration was introduced into the wells, while 120.25, 176.25, and 155.75 mg/mL of the C, S1, and S2 samples, respectively, were applied to yeasts. The positive control wells contained adherent cells and 200 µL of growth medium, while growth medium containing hawthorn extracts was introduced into cell-free wells for the negative control. The plates were then incubated for 24 h at appropriate growth temperatures. After incubation, the supernatant was removed from each well, and the wells were rinsed with physiological saline. A volume of 200 µL methanol was added to each well to fix the biofilm, then the plates were incubated at room temperature for 15 min. Subsequently, methanol was removed and 200 µL of 0.1% (*w*/*v*) crystal violet solution was transferred to the wells. After incubation for 20 min at room temperature, the excess dye was removed by washing the plates with distilled water. Then, the bound crystal violet was released by adding a 33% (*v*/*v*) acetic acid solution. After an incubation step for 10 min at room temperature, the absorbance measurement was performed at 590 nm using a SPECTROstar Nano spectrophotometer (BMG Labtech, Offenburg, Germany). The percentage of biofilm formation was calculated from the optical density data, considering the absorbance of the positive control as 100%.

2.10. Determination of the Anti-Quorum-Sensing Activity of the Extracts

2.10.1. Paper Disk Diffusion Assay

The *C. violaceum* quorum-sensing model organism was used to screen the anti-quorum-sensing capacity of hawthorn extracts in Petri plate incubation. In this method, direct transfer of the testing material is carried out to a paper disk previously placed on the surface of an agar plate inoculated with the biosensor bacterium. First, the LB agar was seeded with 1 mL of *C. violaceum* suspension (10^8 CFU/mL) grown for 24 h in LB broth. A volume of 10 µL from the undiluted extract was impregnated onto a sterile paper disk (5 mm in diameter) previously placed in the middle of the plate. The plates were then incubated for 48 h at 30 °C, and inhibition of quorum sensing was visually evaluated from the uncolored growth halo developed around the paper disk.

2.10.2. Violacein Pigment Extraction Assay

A liquid cultivation test using the *C. violaceum* model organism was also applied to examine the quorum-sensing inhibitory potential of extracts [27]. In this assay, the biosensor bacterium is cultivated in a liquid medium together with the testing material, and the pigment yield obtained is compared to that of a control culture incubated without any inhibitory compound. Briefly, LB broth (9.5 mL) was inoculated with 0.5 mL of 24 h old *C. violaceum* suspension (10^8 CFU/mL), and, except for controls, 100 µL of hawthorn extracts were added to the growth medium. After incubation for 24 h at 30 °C, 2 mL of each sample was centrifuged (16,200× *g* for 10 min), and the pellet was solubilized in 1 mL of dimethyl sulfoxide by vigorous vortexing for 20 min. The samples were then centrifuged (16,200× *g* for 10 min; Heraeus Biofuge Pico, Kendro, Osterode, Germany), and 200 µL of supernatant was pipetted into the wells of a 96-well polystyrene microtiter plate (Sarstedt, Nümbrecht, Germany). Absorbance was measured at 585 nm using a SPECTROstar Nano microplate reader (BMG Labtech, Offenburg, Germany). The pigment production rate was determined from the absorbance data compared to the control and given as a percentage.

2.11. Statistical Analysis

All experiments were performed in triplicate, and average values and standard deviations were determined. Statistical analysis of the results was performed by one-way ANOVA followed by Tukey's multiple comparison test using the GraphPad Prism 8.00 software (GraphPad Software Inc., San Diego, CA, USA). The level of significance was determined by $p < 0.05$. Pearson's correlation coefficients (Pearson r) were calculated using GraphPad Prism 8.00 (GraphPad Software Inc., San Diego, CA, USA).

3. Results and Discussion

3.1. Total Phenolic Content

The hawthorn fruit contains a variety of phenolic compounds such as proanthocyanidins, tannins, flavonoids, and phenolic acids [2]. Many cell wall-bound phenolics and phenolic glycosides (e.g., apigenin glycosides, vitexin rhamnosides) are also found in hawthorn fruit samples, and the bioavailable beneficial properties of these phenolic compounds are limited. In this assay, the cellulase enzymatic support resulted in a positive effect on the free phenolic production, as the TPC increased from 885 ± 13 mg GAE/100 g DM to 1700 ± 128 mg GAE/100 g DM ($p < 0.05$) when the cellulolytic enzyme cocktail of *R. miehei* was used for extraction (Figure 3). The addition of pectinase to the enzyme cocktail further increased the amount of extractable phenolics to 1899 ± 27 mg GAE/100 g DM ($p < 0.05$) (Figure 3).

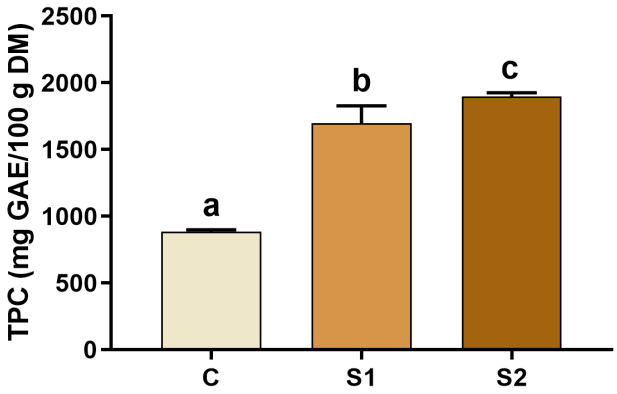

Figure 3. Total phenolic content (TPC) of hawthorn fruit extracts obtained by acetate buffer (C), cellulase (S1) and cellulase–pectinase (S2) extractions. The values shown are averages of the data obtained from three measurements; error bars represent the standard deviation. The different letters above the columns indicate significant differences according to one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

In a previous study, cellulase treatment increased the phenolic content of grape, apple, and pitahaya extracts, and treatment with a combination of cellulase and pectinase further increased the yield of free phenolics for most residues [26]. More recently, carbohydrase treatments were applied efficiently for the extraction of phenolics from other plant residue materials such as sorghum grains [30], the dry extract of *Beta vulgaris* [31], and mulberry fruits [32]. For hawthorn samples, solvent-based treatments have been the most frequent techniques to prepare phenolic-enriched extracts. Alirezalu et al. [5], for instance, used ultrasound extraction in 80% methanol and determined 40.04 mg GAE/g DW of total phenols in *C. orientalis*. Kostić et al. [3] reported 2.12–30.63 mg GAE/g of fresh fruit TPC in *Crataegus oxyacantha*, depending on the solvent extraction methodology used. Treatment with an organic solvent–water mixture was an effective technique for the extraction of phenolics in *C. orientalis* subsp. *orientalis* as well [33]. The 2.86 to 13.81 mg GAE/g DW TPC yield obtained was comparable to those found for *Crataegus microphylla* (2.47–8.63 mg GAE/g DW) [34] and *C. orientalis* (13.36 mg GAE/g DW) [35] achieved with ultrasound or microwave combined solvent extractions. Anyway, our study is the first demonstration that

a carbohydrase enzyme-assisted treatment is also an effective methodology for high-yield phenolic extraction in hawthorn fruit samples.

3.2. Antioxidant Activity

The antioxidant activity of the hawthorn extracts was determined using DPPH and FRAP assays. The FRAP of the sample treated with the *R. miehei* cellulase cocktail improved significantly ($p < 0.05$), presenting an increment of about 1.7-fold after incubation compared to enzyme-free extraction (Figure 4A). An increase (1.15-fold) ($p < 0.05$) was also found in the DPPH scavenging activity when the residues were treated with the *R. miehei* enzyme compared to acetate buffer extraction (Figure 4B).

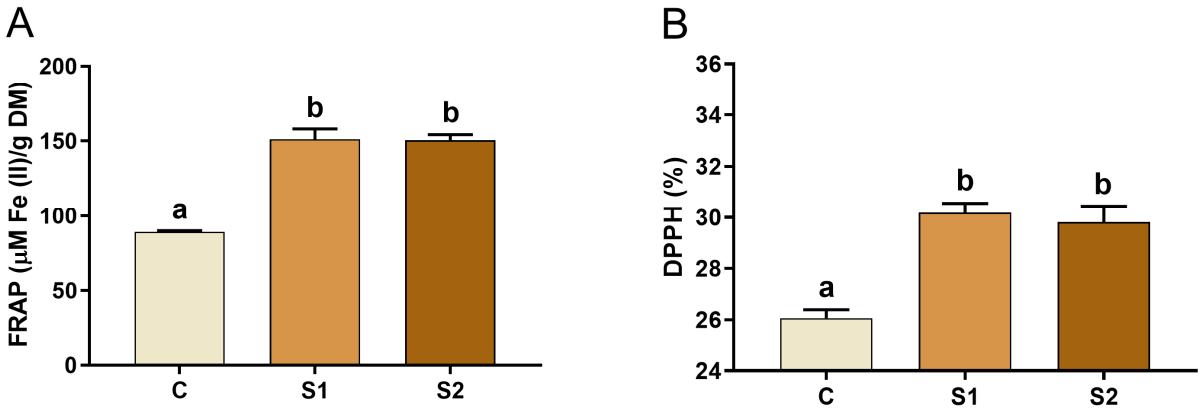

Figure 4. FRAP activity (**A**) and DPPH radical scavenging capacity (**B**) of hawthorn fruit extracts prepared by acetate buffer (C), cellulase (S1), and cellulase–pectinase (S2) extractions. The values shown are averages of the data obtained from three measurements; error bars represent the standard deviation. The different letters above the columns indicate significant differences according to one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

The DPPH radical scavenging activity achieved for the hawthorn extracts (Figure 4B) corresponds to those shown by about 6 μM of gallic acid, catechin, and quercetin or about 32 μM of ascorbic acid in the work of Silva et al. [36]. In addition, the radical scavenging activity exhibited by the extracts (25–30%, Figure 4B) makes them suitable antioxidants in interaction tests [36].

Correlation analysis revealed a strong association between TPC and DPPH inhibition (r = 0.964), as well as FRAP (r = 0.981), suggesting that the phenolics released during enzyme treatments have an important role in the antioxidant activity increase of extracts. A significant positive relationship was also observed between FRAP and DPPH (r = 0.997). Positive relationships between antioxidant activity and phenolic content of extracts have been described in hawthorn [37–39], sorghum [30,40], chestnut [41], and tea [42] samples as well.

It should be noted that even if the measured TPC was increased by adding pectinase to the *R. miehei* enzyme cocktail, the antioxidant capacity was not significantly altered ($p > 0.05$). It is known that the Folin reagent used frequently in TPC measurements is not specific only to phenolics. Other hydroxyl group-bearing compounds, such as reducing sugars, ascorbic acid, tryptophan, and others, can also reduce the reagent [43]. The reducing sugars released from the pectin chain by pectinase hydrolysis may be responsible for the increased Folin reactivity, but these compounds do not contribute to the increase in antioxidant capacity.

The antioxidant activity of various hawthorn extracts has already been published in the literature. For comparison, the level of FRAP was found between 0.34 and 1.84 mmol Fe (II)/g dry weight for fruits of different hawthorn species, including *C. orientalis*, with a value of 0.45 mmol Fe (II)/g dry weight [5]. In *Crataegus monogyna*, the extract of ripened fruits showed an inhibition of about 83% against DPPH radicals, while the inhibitory

effect was about 89% for the unripe fruits [37]. Çalişkan et al. [39] quantified a 21.4% DPPH inhibitory effect in the methanolic extract of *C. orientalis* var. *orientalis*. The *C. orientalis* extract, which also originated from Turkey, demonstrated a DPPH inhibition of 1567.39 half-maximal effective concentration (EC_{50}) in µg/mL equivalents and a FRAP of 0.73 mM $FeSO_4$/g dried extract in the work of Bardakci et al. [38]; the FRAP value was slightly lower to that determined for *C. monogyna* collected from the same geographical area [38]. *C. oxyacantha* extracts produced by Kostić et al. [3] had a percent DPPH inhibitory action between 60.61% and 89.89% depending on the extraction methodology. Although these samples were made via methanol/ethanol-based solvent extraction approaches, antioxidant activity data achieved here with enzyme-assisted extractions proved to also be considerable. In fact, alcohol-based extractions can provide samples with a higher flavonoid content and thus higher antioxidative potential than aqueous methodologies [44]. Enzyme-assisted extractions were carried out mainly in a water-based extraction environment, with decreased use of solvents, which is important to maintain the "green" nature of the process [19,23].

3.3. Individual Phenolics Profile

The LC-HRMS assay showed an increase in concentration of many phenolic compounds, e.g., dihydroxybenzoic acid, protocatechuic acid, caffeic acid, vanillic acid, vanillin, *p*-coumaric acid, ferulic acid, and quercetin, when enzyme-assisted extraction was applied compared to enzyme-free acetate buffer extraction (Table 1). Chlorogenic acid, hyperoside, and isoquercitrin were the dominant phenolic compounds in the sample extracted with acetate buffer, while vanillic acid and ferulic acid were found at the highest concentration in extracts after enzyme treatments (Table 1). The vanillic acid (115.6 times), *p*-hydroxybenzaldehyde (113.5 times), and ferulic acid (93.9 times) demonstrated the largest increase in concentration after the *R. miehei* cellulase enzyme treatment ($p < 0.05$). The caffeic acid content of the *R. miehei* cellulase-treated sample is also worth noting, showing a 16.9-fold increase compared to enzyme-free acetate buffer extraction ($p < 0.05$). The majority of ferulic acid is presented in bound form to fibers in plant residues [45], and the increment in its concentration can be due to the action of degrading enzymes such as cellulases, esterases, and xylanases. Free ferulic acid accumulation was identified, for instance, from wheat bran in the work of Manini et al. [46], which was considered to be the activity of esterases and other fiber-degrading enzymes. The increase in vanillic acid concentration may be the result of lignocellulolytic degradation by the crude *R. miehei* enzyme cocktail and/or by the biotransformation of ferulic acid to vanillic acid [45,47].

The concentration of individual phenolics did not differ significantly ($p > 0.05$) when the extracts of enzyme treatments with *R. miehei* cellulase and *R. miehei* cellulase–*A. niger* pectinase cocktails were compared (Table 1). However, it is worth mentioning that the concentrations of chlorogenic acid, hyperoside, and isoquercitrin compounds in the *R. miehei* cellulase-treated samples were strongly reduced by approximately 5.1, 24.2, and 26.5 times, respectively, compared to enzyme-free extraction (Table 1). This may be attributed to the action of the enzymes found in the cocktails, which caused the degradation of these molecules. For instance, the β-glucosidase activity (2149 U) of the *R. miehei* cellulase cocktail can be responsible for the hydrolysis of the flavonol glucoside compound isoquercitrin. Additionally, the esterases can release caffeic acids from the caffeic acid-quinic acid ester chlorogenic acid, while the β-galactosidases can degrade the phenolic galactoside hyperoside molecule, releasing quercetin during the treatment. Although these latter enzyme activities have not been measured in the enzyme cocktail used, our previous studies revealed considerable esterase and β-galactosidase activities in enzyme cocktails of *R. miehei* produced similarly on wheat bran [30,48]. The enhanced concentration of caffeic acid and quercetin in enzyme-treated extracts could also strengthen this suggestion.

Table 1. Yields of phenolic compounds (in μg/g DM) extracted from *C. orientalis* fruit with acetate buffer solution (C), cellulase (S1), and cellulase–pectinase (S2) cocktails.

Compounds	Type of Extract		
	C	S1	S2
Gallic acid	0.88 ± 0.06 a	2.16 ± 0.21 b	2.07 ± 0.19 b
Dihydroxybenzoic acid	n. d. [1] a	5.00 ± 0.63 b	5.06 ± 0.54 b
Protocatechuic acid	7.54 ± 0.69 a	11.33 ± 0.89 b	10.64 ± 0.91 b
(+)-Catechin	0.18 ± 0.02 a	0.03 ± 0.01 b	0.04 ± 0.01 b
Protocatechuic aldehyde	0.59 ± 0.05 a	0.52 ± 0.03 a	0.53 ± 0.02 a
Chlorogenic acid	94.03 ± 7.15 a	18.43 ± 2.11 b	19.85 ± 3.25 b
(−)-Epicatechin	4.30 ± 0.08 a	1.64 ± 0.12 b	1.58 ± 0.17 b
Caffeic acid	1.28 ± 0.37 a	21.66 ± 3.44 b	21.87 ± 4.11 b
Vanillic acid	3.47 ± 0.61 a	401.20 ± 47.13 b	441.85 ± 53.75 b
Syringic acid	0.67 ± 0.05 a	3.76 ± 0.66 b	3.79 ± 0.54 b
p-Hydroxybenzaldehyde	0.02 ± 0.00 a	2.27 ± 0.16 b	2.31 ± 0.18 b
Vanillin	8.53 ± 0.80 a	13.68 ± 1.12 b	13.92 ± 1.35 b
Taxifolin	0.07 ± 0.02 a	0.04 ± 0.01 a	0.04 ± 0.01 a
Vitexin	3.64 ± 0.91 a	1.39 ± 0.11 b	1.45 ± 0.16 b
p-Coumaric acid	4.24 ± 0.07 a	11.91 ± 1.63 b	11.59 ± 2.11 b
Ferulic acid	1.71 ± 0.80 a	160.66 ± 19.82 b	154.89 ± 21.26 b
Isovitexin	2.66 ± 0.94 a	0.96 ± 0.21 b	0.95 ± 0.19 b
Hyperoside	99.31 ± 3.56 a	4.09 ± 1.21 b	4.31 ± 1.62 b
Isoquercitrin	22.57 ± 1.06 a	0.85 ± 0.23 b	0.96 ± 0.16 b
Quercetin	5.85 ± 0.94 a	12.70 ± 1.02 b	11.66 ± 1.61 b
Apigenin	0.06 ± 0.01 a	0.01 ± 0.00 b	n. d. b

[1] Not detected. The values shown are averages of the concentration data obtained from three measurements ± standard deviation. The different letters within a row indicate significant differences according to one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

The individual phenolic profile can vary from hawthorn residue to residue. For fruit samples, chlorogenic acid, hyperoside, and isoquercetin compounds were generally identified as the main phenolics in many studies [5,38,49]. Epicatechin, catechin, quercetin, and rutin were also dominant compounds in *Crataegus pubescens*, *Crataegus azarolus*, and *C. monogyna* fruit extracts [50–52]. For *C. orientalis*, Alirezalu et al. [5] quantified chlorogenic acid, rutin, hyperoside, isoquercetin, and quercetin in fruit samples from Iran. (−)-Epicatechin, (+)-catechin, quercetin, chlorogenic acid, vitexin, hyperoside, rutin, and/or *p*-coumaric acid were detected as dominant phenolic compounds in fruit samples of *C. orientalis* collected from Turkey [33,35,38]. Many of these compounds, as well as isoquercitrin, were also identified in the *C. orientalis* berry extract from Macedonia [53]. Except for rutin, these phenolic molecules were also detected in *C. orientalis* fruit residue extracts prepared with both acetate buffer and enzyme-assisted extractions in this study (Table 1).

3.4. Antimicrobial Capacity of Hawthorn Extracts

Hawthorn fruit extracts could show an antimicrobial effect according to previous studies [2], and the effectiveness of which may vary depending on the mode of extraction. In line with this, the growth inhibitory potential of hawthorn fruit extracts prepared by acetate buffer and enzyme-assisted extractions was studied against *E. coli*, *P. aeruginosa*, *B. subtilis* and *C. violaceum* bacteria, and *W. anomalus* and *D. hansenii* yeasts. The results for MIC of the hawthorn fruit extracts are summarized in Table 2. As can be seen, the antimicrobial effect varied depending on the extract and microorganism during the applied microdilution tests. The results showed a MIC for each extract against the bacteria, while the yeasts tested were quite resistant to the activity of the extracts.

Table 2. Minimum inhibitory concentration (MIC) of *C. orientalis* fruit extracts prepared with acetate buffer extraction (C), cellulase (S1), and cellulase–pectinase (S2) treatments against selected bacteria and yeasts.

Extracts	MIC (mg/mL)					
	E. coli	*P. aeruginosa*	*B. subtilis*	*C. violaceum*	*W. anomalus*	*D. hansenii*
C	15.0	30.1	30.1	15.0	120.2	>120.2
S1	22.0	44.1	44.1	88.1	>176.2	>176.2
S2	38.9	38.9	38.9	77.9	>155.7	>155.7

In the case of *P. aeruginosa* and *B. subtilis*, the MICs were identified at the second step of dilution of extracts, i.e., in 4-fold diluted samples, regardless of the extraction method (Table 2). MIC against *E. coli* was also obtained in a 4-fold dilution when the extract produced by the combined treatment of cellulase–pectinase was used in the tests. However, in *E. coli*, lower concentrations for extracts of acetate buffer and cellulase treatment were also active against planktonic growth, resulting in MIC in the 8-fold diluted samples (Table 2). *C. violaceum* growth was less sensitive to extracts compared to other bacteria tested. Although the MIC against *C. violaceum* was identified in the 8-fold diluted sample of the acetate buffer extract, the same inhibitory effect was observed at higher sample concentrations in enzyme-treated extracts (Table 2).

Unlike the notable increase in antioxidant capacity, the enzyme-assisted treatment did not result in enhanced antimicrobial activity for hawthorn fruit extracts compared to acetate buffer extraction (Table 2). Even though the increased yield of catechin and ferulic acid after enzyme treatment (Table 1) may exert an oxidative stress-inducing effect in bacteria, which may result in an increased antimicrobial effect [54]. In fact, different bacteria can show different sensitivity to extracts, and the increased carbohydrate oligomer content of extracts produced by enzymatic treatments can support the bacterial growth, as has been described for the formation of bacterial biofilms as well [27]. Additionally, it was also reported that the concentration of certain phenolic compounds is not necessarily responsible for the antimicrobial effect [27]. An inhibitory effect may also be due to synergy between phenolics [55,56] and/or the presence of other phenols not detected in our tests. Anyway, both the samples extracted with acetate buffer and the samples treated with carbohydrase enzymes can be considered as growth inhibitors for the tested bacteria. It is worth noting, however, that the inhibitory effect of the obtained hawthorn extracts was weak considering the MIC value ranges reported by Tamokou et al. [57] for edible plant extracts potentially used in the treatment of infectious and systemic diseases. For further comparison, the growth of the *E. coli*, *P. aeruginosa*, and *B. subtilis* strains involved in this study was strongly inhibited by 1 µg/disc streptomycin in our previous assay [27]. However, 10 µg/disc amounts of phenolic acid, flavonoid, and stilbene compounds were not effective against the growth of these isolates, and the MIC values for the phenolics tested ranged between 125 and >500 µg/mL [27]. The inhibitory effect of hawthorn samples could be ascribed to the direct interaction of polyphenolics in extracts with the cell membrane, causing its damage and, thereby, the leakage of intracellular substances and/or the intake of extracellular inhibitor compounds such as phenolic molecules [4].

Crataegus extracts have been documented to be potential antimicrobial agents against a variety of bacteria. Ghendov-Moşanu et al. [58], for instance, determined a 62.5 mg/mL MIC value for *C. monogyna* aqueous ethanolic extracts against *E. coli*. The solvent extracts of *C. monogyna* fruit were also effective against bacteria such as *P. aeruginosa*, *B. subtilis*, *E. coli*, and *Staphylococcus aureus*, with MIC values between 0.625 mg/mL and >10 mg/mL depending on the solvent used for extraction [59]. *C. oxyacantha* in 10 mg/mL concentration exhibited an inhibition zone of 22 and 27 mm against *E. coli* and *P. aeruginosa*, respectively, but no inhibition was determined against *B. subtilis* [3]. Considerable inhibition was reported against *S. aureus* and *Streptococcus faecalis* for *C. azarolus* leaves and fruit peel extracts in the study of Belkhir et al. [60]. A strong antimicrobial action against the growth

of *B. cereus*, *S. aureus*, *E. coli*, and *Salmonella enterica* with MICs of 2.5, 5, and 10–10 mg/mL, respectively, was also identified for a pulp extract of *Crataegus elbursensis* prepared with methanol [61]. A *C. elbursensis* fruit extract showed 97% and 84% growth-reducing effects toward *E. coli* and *S. aureus*, respectively, in the work of Safapour et al. [62].

The tested *W. anomalus* and *D. hansenii* yeasts showed a high resistance against both enzyme-free and enzyme-treated hawthorn extracts (Table 2). No MIC was found against *D. hansenii* even at the highest fruit extract concentrations used. However, in *D. hansenii*, growth inhibitory effects of 32%, 42%, and 46% were registered for extracts prepared with acetate buffer, cellulase, and cellulase–pectinase treatments, respectively, at the highest test concentration of each extract. In *W. anomalus*, the MIC was achieved for the acetate buffer extract at the highest concentration (Table 2). The extract prepared by cellulase-assisted treatment exhibited a 12% inhibition, while the sample obtained by cellulase–pectinase treatment had even lower inhibition (8%) at the same concentration. In fact, yeasts are commonly present on the surface of different fruits [63,64]. In line with this, some of them could show tolerance to the inhibitory effect of phenolic compounds due to the volatile phenols present in the fruit [65]. Anyway, the planktonic growth of *W. anomalus* and *D. hansenii* was only slightly inhibited by many phenolic compounds, i.e., hydroxybenzoates, hydroxycinnamates, stilbenes, flavonoids, and phenolic aldehydes, in a recent assay [66]. In that study, the MICs of the tested phenolic compounds were between 0.5 and >2 mg/mL [66]. However, in *Candida albicans*, Kostić et al. [3] reported 13- and 20-mm growth inhibition zones for the *C. oxyacantha* fruit extract. The extracts of *C. monogyna* fruit were also effective against *C. albicans*, presenting MICs between 0.625 and 2.5 mg/mL depending on the mode of sample preparation [59].

3.5. Influence of Hawthorn Extracts Against Biofilms

In this assay, five common food-contaminating microorganisms, namely *E. coli*, *P. aeruginosa*, *B. subtilis*, *W. anomalus*, and *D. hansenii* involved in the antimicrobial tests, were subjected to antibiofilm effect analysis. None of the hawthorn extracts tested showed an inhibitory effect against the formation of *E. coli* and *P. aeruginosa* biofilms (Table 3). In *B. subtilis*, extracts prepared by enzyme-assisted treatment resulted in a significant ($p < 0.05$) reduction in biofilm mass compared to positive control; however, the effect varied depending on the type of enzyme treatment (Table 3). Namely, about 15.1% inhibition was registered against the growth of the *B. subtilis* biofilm when the hawthorn extract treated with *R. miehei* cellulase was added, while the biofilm formation was reduced by about 84.4% in the presence of the extract treated with *R. miehei* cellulase–*A. niger* pectinase, compared to the corresponding enzyme-free sample. Overall, the highest inhibitory effect was registered in *B. subtilis*. In this context, previous studies also identified phenolic-enriched plant extracts, e.g., solvent extracts of olive leaves [67], *Potentilla visianii* [68], and pomegranate peel [69], as efficient agents against the growth of *B. subtilis* biofilms.

Table 3. Effect of acetate buffer (C)-, and cellulase (S1)-, and cellulase–pectinase (S2)-treated hawthorn fruit residues on the biofilm formation (%) of food-contaminating bacteria. The biofilm formation measured in the absence of any extract was taken as the positive control (100%).

Extracts	Biofilm Formation (%)		
	E. coli	*P. aeruginosa*	*B. subtilis*
Positive control	100 ± 0 a	100 ± 0 a	100 ± 0 a
C	105.4 ± 1.4 b	98.6 ± 1.7 a	98.3 ± 2.0 a
S1	101.6 ± 2.2 a	99.3 ± 3.1 a	84.9 ± 3.2 b
S2	104.5 ± 0.7 a	98.8 ± 0.3 a	15.6 ± 1.7 c

MIC/2 concentrations were used for the corresponding extract. The values shown are averages of the percent biofilm growth data obtained from three measurements ± standard deviation. The letters within columns indicate significant differences between mean values; one-way ANOVA, Tukey's multiple comparison test ($p < 0.05$).

The biofilm growth of yeasts was generally more sensitive to the extracts than that of bacteria. The biofilm growth of *W. anomalus* was significantly ($p < 0.05$) decreased with inhibitory effects of 36% to 43% in the presence of the extracts, irrespectively of the extraction method (Figure 5). In *D. hansenii*, the decreasing effects of the cellulase and cellulase–pectinase-treated extracts against biofilm formation were 61% and 52%, respectively. However, no significant inhibition towards biofilm growth was registered for the acetate buffer-extracted sample compared to the extract-free positive control ($p > 0.05$) (Figure 5). As can be seen, the enzyme treatments increased the anti-biofilm formation effect of hawthorn fruit extracts in *D. hansenii* compared to the inhibitory data from the acetate buffer-extracted sample (Figure 5). The *W. anomalus* and *D. hansenii* biofilms were sensitive to the presence of some individual phenolics, e.g., vanillin, vanillic acid, ferulic acid, or quercetin, in the study of Kimani et al. [66]. For comparison, a considerable increase was recorded in the concentration of most of these biofilm inhibitor phenolic compounds after the enzymatic treatment of the hawthorn residue (see Table 1).

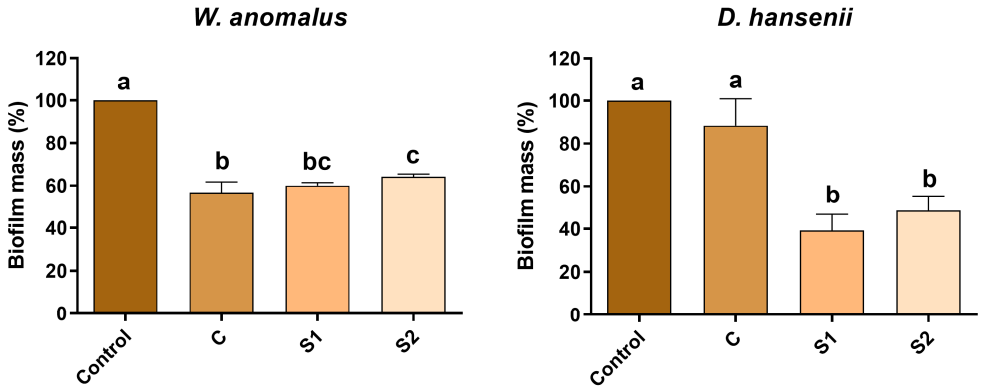

Figure 5. Inhibitory capacity of hawthorn fruit extracts prepared by acetate buffer or via enzyme-assisted extraction against *W. anomalus* and *D. hansenii* yeast biofilms. C–acetate buffer extraction, S1–extraction with *R. miehei* cellulase cocktail, S2–extraction with pectinase supplemented *R. miehei* cellulase cocktail. Extracts with concentrations of 120.25 (C), 176.25 (S1) and 155.75 (S2) mg/mL were used in the tests. The control (Control) represents the biofilm formation in the absence of hawthorn residue extract. The values shown are averages of the data obtained from three measurements; error bars represent the standard deviation. The different letters above the columns indicate significant differences according to one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

The biofilm inhibitory potential of hawthorn extracts has been less investigated. In the study of Zhang et al. [4], the upregulation of genes related to biofilm formation was identified in the presence of a phenolic-rich solvent extract of *Crataegus pinnatifida* Bge. The increased expression of these stress response and oxidative stress genes may be related to cell oxidative and environmental stress caused by the hawthorn extract [4]. No IC_{50} was identified against methicillin-resistant *S. aureus* biofilms by Quave et al. [70] even at 128 µg/mL concentration for ethanolic extracts from leaves, stems, and flowers of *Crataegus monogyna* Jacq. Anyway, the results presented here show for the first time that *C. orientalis* fruit extracts prepared by enzyme-assisted extraction are promising substances capable of reducing the growth of bacterial and yeast biofilms.

3.6. Anti-Quorum-Sensing Activity

The inhibitory activity of the hawthorn extracts against quorum sensing was studied using the *C. violaceum* 85WT biosensor bacterium. The violacein pigment synthesis in the model organism is regulated by the quorum-sensing signaling system, and test extracts that can inhibit the pigment production are supposed to be samples that could affect the regulation process [29].

The paper disk assay showed a quorum-sensing inhibitory effect for enzyme-free extracts made with acetate buffer extraction. The formation of a non-purple ring around the disk indicated bacterial growth with reduced violacein pigment production (Figure 6A). Both samples prepared via enzyme-assisted extraction did not result in any pigment-free ring around the disks. However, in the liquid culture assay, a significant ($p < 0.05$) reduction in the violacein production was registered for the samples after carbohydrase treatments as well compared to control (Figure 6B). The violacein production was 90.3%, 87.2%, and 90.9% in the presence of acetate buffer extract and *R. miehei* cellulase and *R. miehei* cellulase–*A. niger* pectinase-treated extracts, respectively. Treatment with *R. miehei* cellulase significantly stimulated ($p < 0.05$) the anti-quorum-sensing activity of the hawthorn residue compared to the enzyme-free extract.

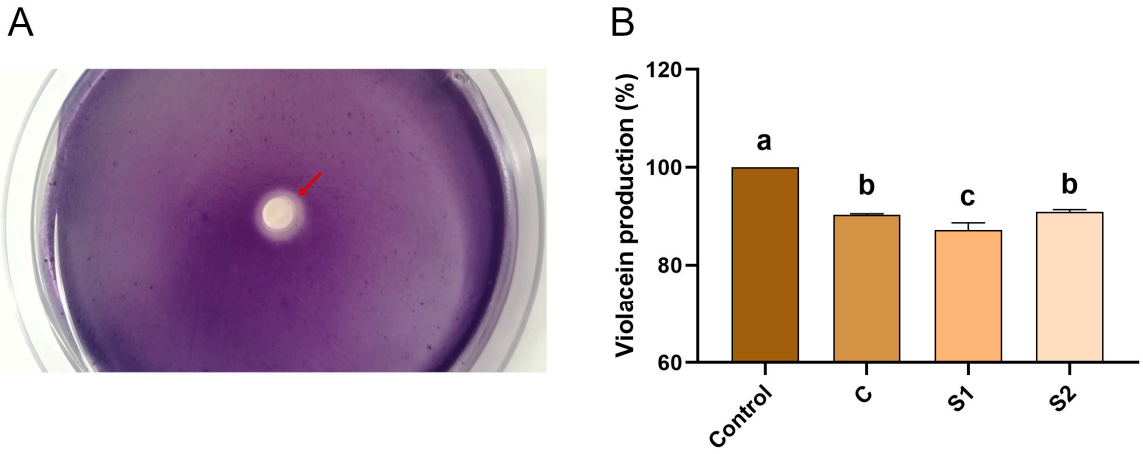

Figure 6. Inhibition of violacein production in *C. violaceum* 85WT quorum-sensing biosensor bacterium by hawthorn extracts. (**A**) Reduced pigment synthesis around the disk impregnated with acetate buffer extracted hawthorn sample (1.2 mg/disk). The red arrow indicates the non-purple ring around the disk. (**B**) Percent violacein production in liquid culture assay in the presence of hawthorn extracts prepared by acetate buffer (C, 1.2 mg/mL reaction mixture), *R. miehei* cellulase (S1, 1.7 mg/mL reaction mixture) and *R. miehei* cellulase–*A. niger* pectinase (S2, 1.5 mg/mL reaction mixture) extractions. Violacein production measured in the absence of the given extract was taken as 100% (Control). The values shown are averages of the data obtained from three measurements; error bars represent the standard deviation. The different letters above the columns indicate significant differences according to one-way ANOVA followed by Tukey's multiple comparison test ($p < 0.05$).

Anti-quorum-sensing activity has been demonstrated for other plant extracts as well, including those prepared from various medicinal plant and fruit residue samples [27,71–75]. Natural antioxidative phenolic compounds could also show an inhibitory effect against bacterial quorum sensing [75,76]. However, relatively few reports are available on the anti-quorum-sensing activity of hawthorn samples. Ethanolic extract of *C. monogyna* Jacq. leaves, stems, and flowers could efficiently inhibit the δ-toxin production of methicillin-resistant *S. aureus* (MRSA) [77]. The resulted 57% inhibition of toxin production indicated a moderate anti-quorum-sensing activity in this pathogenic bacterium [77]. A 14.2 mm pigment-free halo was identified for the acetone-water extract of the *Crataegus cuneata* fruit in the study of Yeo and Tham [78], using a disk diffusion test based on a *C. violaceum* CV026 biosensor. In fact, no study has reported to date that cellulase treatment increases the quorum-sensing inhibitory potential of hawthorn residue samples.

4. Conclusions

This study demonstrated the phenolic content, antioxidant activity, and biological properties of hawthorn fruit extracts prepared via enzyme-assisted treatments by *R. miehei* cellulase and *R. miehei* cellulase–*A. niger* pectinase cocktails. The extracts obtained by

enzyme treatments showed a higher phenolic content and antioxidant activity compared to those prepared by enzyme-free extraction. Chromatography revealed an improvement in the concentration of individual phenolics as a result of the enzyme treatments, and the largest yield development was identified for vanillic acid, *p*-hydroxybenzaldehyde, and ferulic acid compounds. The quantity of chlorogenic acid, hyperoside, and isoquercitrin markedly decreased during both cellulase and cellulase–pectinase treatments. The extracts exhibited moderate antimicrobial activity against planktonic growth of *E. coli*, *B. subtilis*, *P. aeruginosa*, and *C. violaceum* bacteria, while the tested *W. anomalus* and *D. hansenii* yeasts were quite resistant, irrespectively of the residue treatment methodology. The enzyme-treated extracts had biofilm growth inhibitory activity in *B. subtilis*, *W. anomalus*, and *D. hansenii*, as well as inhibited the quorum-sensing mechanism of *C. violaceum*.

Although the hawthorn extracts produced in this study exhibited moderate antioxidant and antimicrobial activities, it can be concluded that carbohydrase-assisted extraction is a reliable strategy for the release of free phenolics from hawthorn fruit residues. To our knowledge, this is the first demonstration of the phenolic profile and biological properties of extracts produced by enzymatic treatments from hawthorn fruit samples. These phenolic-enriched hawthorn extracts have potential applications in the food industry as a natural source of aroma compounds, precursors of aroma substances, and antioxidative preservatives, particularly in functional beverages, purees, and jams, to enhance nutritional value.

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