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Chemical Composition and Antifungal Activity of Coffee By-Products and Chitosan Incorporated into a Polymeric Matrix on *Botrytis* sp. and *Rhizopus* sp.

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Abstract: In blueberry commercialization, non-biodegradable synthetic plastic packaging is used for refrigerated storage to maintain the quality of the horticultural product. The fungi *Botrytis* sp. and *Rhizopus* sp. can cause significant losses at this stage. Consequently, the formulations and production of degradable polymeric based on polylactic acid (PLA)/poly(butylene adipate-co-terephthalate) (PBAT) 60/40 (PP) with coffee parchment (CP), green coffee bean oil (GCBO), chitosan solution (Ch), chitosan nanoparticles (ChNp) and nanostructured coating (NC) were used to develop biodegradable polymer matrix (PM). Caffeine and hexadecanoic acid were identified as major compounds in GCBO, and the major compounds in CP were flavonoids, terpenes and lignans. The 100% of mycelial growth inhibition was observed with 1, 2 and 3% of GCBO, to *Botrytis* sp. and 20% Ch, 10 and 20% ChNp, 20% NC on *Botrytis* sp. and *Rhizopus* sp.; the GCBO inhibited 100% of spore production in both fungi at all evaluated doses. In the in vivo tests, for *Botrytis* sp. a lower incidence of 30% and a severity of 20% was obtained with the treatment of NC compared to the control. For *Rhizopus* sp., an incidence of 55% and a severity of 10% were observed with NC. The PM in the culture medium presented a fungistatic effect. The greatest inhibition of mycelial growth (63%) on *Botrytis* sp. was with PLA/PBAT+Nano coating (PP+NC) and (100%) was observed with PM+CW made with PLA/PBAT+CP+NC (PPCP+NC), PP and PP+NC on *Rhizopus* sp. Coffee by-products and PM have potential as natural fungicides for the control of postharvest fungi in fruits and vegetables.

Keywords: *Coffea* spp; agricultural residues; antimicrobial activity; bioactive compounds; blueberry; chitosan nanoparticles

1. Introduction

Blueberry (*Vaccinium corymbosum*) is a fruit with high nutritional value, low caloric content, high antioxidant content and anti-inflammatory properties [1]. These attributes make it an attractive product due to its versatility and benefits for human health. For these reasons, its popularity and demand have increased in the last decade, increasing production and cultivated areas in different regions of the world. Fungal diseases are the main causes of the reduction in plant productivity. The most recurrent genera are *Botrytis* and *Rhizopus*, which affect the fruit in the post-harvest stage [2]. In blueberries, losses of up to 30% in total production have been recorded due to these phytopathogens [3]. Infections caused by *Botrytis* sp. are probably the most common worldwide, with a wide distribution and a wide range of crops. The disease it causes tends to be more severe in cold and humid environments; this fungus can develop even at 0°C. *Rhizopus* sp. causes soft rot in most fleshy fruit during storage and marketing; when growing conditions are favorable, it can cause large losses in a short period of time due to its rapid development [4]. Today, there are several methods for combating disease-causing agents in agriculturally important crops; however, indiscriminate pesticide use has led to the creation of new biological alternatives that help combat this problem [5]. One of these proposals is to use compounds of natural origin; several plants contain bioactive agents that act against disease development in agriculturally important crops [6]. On the other hand, to the high production of coffee, there is a large quantity of waste that has a wide application due to its physical and chemical properties, bioavailability, and potential in food production, making it useful in various fields [7].

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Green coffee bean oil is obtained from unroasted beans and has several applications in both the cosmetic and food industries due to the presence of bioactive compounds, such as polyphenols, tocopherols, and phytosterols [8]. The (CP) is the covering of the bean at the parchment stage and is considered an agro-industrial waste because it does not have a very demanded use. Recently, it has been sought to explore new applications because CP is a by-product rich in lignocellulosic matter and contains antimicrobial compounds, such as alkaloids and flavonoids, that function as a defense against coffee pathogens and pests [9]. Extracts and oils can be obtained by conventional extraction methods and solvents [10]. Gloria et al. [11] attributed the antimicrobial activity of coffee to caffeine, trigoline, phenolic acids and their derivatives, and after the bean roasting process, it was attributed to melanoids and dicarbonyl compounds. Generally, the bioactive compounds present in coffee are chlorogenic acids, quinic acid, malic acid and caffeine [12]. Another naturally occurring compound is chitosan, which is obtained from the chitin of some mollusks ([13]. It has a high impact on the use of biopolymers and green chemistry due to its high antimicrobial activity. This property depends on several factors such as the type of pathogen, pH of the medium, and the structure and concentration of chitosan [14]. One of the main uses of chitosan is in the preparation of coatings for fruit and vegetable preservation; they help to reduce deterioration due to the presence of microorganisms and maintain fruit quality [15]. Sotelo-Boyás et al. [16] found that using chitosan nanoparticles enhanced their antimicrobial effects because the nanoparticles could more easily penetrate the wall of the microorganisms. Other polymers used to produce packaging are of synthetic origin, such as polyethylene terephthalate, polystyrene and polypropylene, which have a negative impact on the environment and health. Today, there is great interest in generating polymers of natural origin that are biodegradable and compostable [17]. Polylactic acid (PLA) is a biodegradable polymer because it is a derivative of lactic acid obtained from renewable resources, such as corn starch and sugar cane, which makes it a polymer of great importance today [18]. Poly(butylene adipate-co-terephthalate) (PBAT) is a synthetic polymer obtained from fossil-based resources; however, it has the capacity to be biodegradable and has the potential to be used in various applications [19]. More knowledge of the management and handling of plastics is needed since the main problem they have had is due to a lack of information on their origin and adequate treatment, either recycling or composting for their reintegration [20]. Currently, in the food industry, there is a search for incorporating bioactive compounds of natural origin to improve the organoleptic characteristics of fruit and vegetable products, extend their shelf life, and prevent their deterioration by external agents, such as microorganisms [21]. This study had aimed (i) to identify chemical compounds of GCBO and CP using chromatographic techniques; (ii) investigating the antimicrobial activity of individual coffee residues in vitro and in vivo against *Botrytis* sp. and *Rhizopus* sp.; and (iii) evaluating into a biodegradable polymeric matrix (PM) in vitro.

2. Materials and Methods

2.1. Materials

The CP was provided by the coffee producers of Coatepec Veracruz, Mexico. The residues were cleaned and separated manually to eliminate external agents. They were then subjected to a drying process using an oven (Binder FD 115, Tuttlingen, Germany) at 60°C for 12 h to eliminate the total content of humidity. After drying, grinding was carried out in a pulverizing mill (INMIMEX M-150, Tlaxcala, Mexico) and the dust particles that passed through a 100 mesh sieve were recovered. The powders were stored in a container until use. To obtain the oil, ground green coffee beans were used and sifted through a 60-mesh sieve. The Soxhlet extraction system used 50 g of sample placed in the cartridge and left for 6 h in the reflux system with hexane. The extracted oil was recovered and then concentrated in a rotary evaporator (BUCHI R-300, Flawil, Switzerland) to eliminate solvent residues. The sample was packaged and stored in a refrigerator.

2.2. Characterization of coffee oil by Gas chromatography and mass spectrometry (GC-MS)

To identify compounds from green coffee beans oil using gas–mass chromatography, (Agilent Technologies 890B Gas Chromatograph California, USA), with a thermal separation probe (Agilent G4381A-TPS) and 0.6 mg of oil was placed in a 50-µL TSP microvial and analyzed. The equipment was operated under the following conditions: helium as carrier gas, an initial temperature of 80°C, a final temperature of 300°C, an injector temperature at 250°C, and a run time of 45 min. The mass detector operated under the following conditions: mass range of 32–750 atomic mass units (MS), at a transfer temperature of 280°C, and with a

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solvent delay of 0.2 min. The identification of the major compounds was carried out based on the spectra of the NIST library.

2.3. Characterization of CP by Liquid chromatography and mass spectrometry (HPLC-MS)

To identify CP compounds, 1 g of husk was deposited in 10 mL of methanol, and 100 µL was diluted in 900 µL of distilled water and directly injected (10 µL) into a Chromatograph (HPLC) Ultimate 3000 (Dionex Corp, CA, USA) equipped with an array of diodes and a micrOTOF Q-IIanalyzer in electrospray ionization (ESI) system mode (Bruker Daltonics, Billerica, MA) [22]. To identify the compounds, MzCloud, MassBank and bibliographic databases.

2.4. Environmental scanning electron microscopy (ESEM-EDS)

For elemental analysis of CP, the samples were placed on aluminum stubs with double-sided carbon conductive tape and were observed directly under an environmental scanning electron microscope (Carl Zeiss, EVO LS10, Germany) with an acceleration voltage of 30 kV and a pressure of 90 Pa. A backscattered electron detector (NTS BSD) was used, and images were obtained in grayscale and stored in TIFF format with a resolution of 1044 × 756 pixels.

2.5. Nanoparticles and nanostructured coating elaboration

The nanoparticles were prepared using the nanoprecipitation method, which consisted of using a solvent phase composed of 96% ethanol and 0.1 mL of Tween 20 for every 100 mL of ethanol. In addition, a second phase composed of a 0.05% chitosan solution (América Alimentos, Guadalajara, Mexico); 6.25 mL per 100 mL of ethanol, pH 5.6) was used. The chitosan had an intrinsic viscosity of 440.03 ± 6.92 mL·g^{-1}, a molecular weight of 89,305.66 ± 1850.49 g·Mol^{-1} and a degree of deacetylation of 89.44 ± 0.31%. Nanoparticle formation and concentration were determined according to the method proposed by Istúriz-Zapata et al. [23] and were previously characterized by Istúriz-Zapata et al. [24]. To prepare the chitosan coating with the nanoparticle solution, it was mixed in a 9:1 ratio with a 1% chitosan solution (pH 5.6) and a homogenizer (Virtis, Gardier, USA) at 20,000 rpm for 20 min.

2.6. Incorporation of coffee waste and NC in a polymeric matrix

To produce PM, mixtures of PLA and PBAT polymers were pelletized in a 60/40 ratio, according to Correa-Pacheco et al. [25], who mentioned that the mixture of these polymers improved the physical characteristics, such as greater flexibility and an increase in the elongation capacity at break. In addition, 5% CP since it was the optimal loading point for better physical characteristics. To obtain the PM, an extrusion process was carried out using a twin-screw extruder (Process 11, Thermo Scientific TM, MA, USA), which had eight heating zones, a die and four feed ports. The following temperatures were used: 160/180/180/180/180/170/170/150 and the die at 160°C. The PLA/PBAT 60/40 pellets with 5% CP were previously dried in a vacuum oven at 60°C for 24 hours to remove moisture. These were placed in the first feed port while the GCBO was incorporated into the second feed port using a peristaltic pump (MasterFlex C/L, Cole-Parmer, IL, USA). At an injection speed of 0.2 rpm, the purpose of incorporating GCBO was to take advantage of its antimicrobial and plasticizing properties that provide greater flexibility, lower rigidity and crystallization, improving the mechanical properties of the polymer [26]. Two variables were used in the cooling bath: one was to use water and the second was to use NC to adhere to the PM.

2.7. Fungal genera

Botrytis sp. and *Rhizopus* sp. were obtained from the collection of the CEPROBI-IPN Postharvest Technologies Laboratory, identified by their morphological characteristics and reactivated in potato dextrose agar culture medium. The Petri dishes were incubated to 8 days for *Botrytis* sp. at 12°C ± 2 and 28°C ± 2 for 43 h for *Rhizopus* sp.

2.8. Mycelial growth inhibition

The method reported by Velázquez Silva et al. [27] was used for the evaluation. The treatments consisted of: control potato dextrose agar (PDA), CP (1, 5 and 10%), GCBO (1, 3 and 6%), NC (1, 10 and 20%), Ch (1, 10 and 20%) and ChNp (1, 10 and 20%). Milliliters six of each mixture was added to 9 cm diameter Petri dishes. Once the culture medium solidified, 10 µL of a spore suspension (1 × 10^{-5}) of *Botrytis* sp. and *Rhizopus* sp. was placed in the center of the dish and incubated under the conditions mentioned above. The radial growth of the fungus was measured using a Vernier caliper (Cienceware, CA, USA) until the control treatment covered 100% of the Petri dish. The results were reported in millimeters (mm). Six repetitions were

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carried out per treatment (n=6), and at the end of the test, the percentage of mycelial inhibition was calculated using the following equation:

$$\%MI = [(A-B)/A] \times 100, \quad (1)$$

where A is the mycelial growth of the control group and B is the mycelial growth of the pathogen in the applied treatments.

The growth rate was calculated using the equation:

$$Gr = [(DF-DI)/(FT-IT)], \quad (2)$$

where Gr is the growth rate, DF is the diameter of the final growth DI is the diameter of the initial growth, FT is the final time of mycelial growth in days or hours, and IT is the start time.

For percentage data, a Bliss angular transformation was used using the following equation:

$$y^* = \text{arcsen}\sqrt{(y/100)} \quad (3)$$

2.9. Spore germination inhibition

The mycelium was scraped with sterile water and filtered to obtain a spore suspension of each treatment. Three discs of 1 cm diameter PDA medium were placed on a sterile slide; the discs were inoculated with 20 µL of the spore suspension. Spore germination was stopped by applying 80% lactic acid at 4, 6 and 8 h, and the number of germinated spores was counted. The percentage of spore germination inhibition was calculated using the equation:

$$SGI\ (\%) = [(SG\ Control - SG\ Treatment)/SG\ Control] \times 100 \quad (4)$$

where SGI is the spore germination inhibition in percentage and SG are the germinated spores.

2.10. In vivo evaluation

Blueberry fruit (variety Sweetest batch) collected from Zapopan, Jalisco, Mexico, was used. The fruit was disinfected with a 400 ppm sodium hypochlorite solution for 3 min and rinsed with sterile distilled water. The 1×10^{-5} spore suspension was prepared for the fungi *Botrytis* sp. and *Rhizopus* sp., and 10 µL of the suspension was placed on each fruit previously punctured with a sterile dissection needle. The fruit was immersed for 1 minute in different treatments for evaluation. For ground pure CP, a 1:1 portion of CP and sterile distilled water were used and compared with a commercial fungicide (CAPTAN 500 MezFer) at 3%. Fifteen fruits were used for each treatment, and each treatment was placed in a PET clamshell and stored at room temperature (28±2°C) under relative humidity conditions (95±2%). Evaluations were made every day, and severity and incidence were determined using the equation:

$$\%Incidence = (Number\ of\ infected\ fruits/Total\ number\ of\ fruits) \times 100 \quad (5)$$

Severity was determined by the percentage of fruit damage.

2.11. In vitro assay for the PM

The method described by Correa-Pacheco et al. [28] was used with modifications. A 5 mm circle of the PM was placed in the center of the Petri dish with PDA medium, and 10 µL of the 1×10^{-5} spore suspension of *Botrytis* sp. and *Rhizopus* sp. was added. The radial growth of the fungus was measured using a Vernier caliper (Cienceware, CA, USA) until it covered 100% of the Petri dish in the control treatment. Six repetitions were carried out per treatment (n=6), and at the end of the incubation time, mycelial growth was reported. The treatments evaluated in this trial were PLA/PBAT (PP), PLA/PBAT with chitosan coating (PP+NC), PLA/PBAT with CP (PPCP), PLA/PBAT with CP and chitosan coating (PPCP+NC), and Polyethylene Terephthalate (PET).

2.12. Statistical analysis

In vitro results were analyzed by a one-way ANOVA) with Tukey's comparison of means ($p<0.05$). InfoStat software version 2020 was used.

3. Results

3.1. Characterization of green coffee bean oil by GC-MS

Six compounds were identified in green coffee bean oil. Figure 1 shows the detection peaks and Table 1 shows the following composition: 10% caffeine alkaloid, 26.5% fatty acids, 30% methylated aromatic compounds, and 20.5% hydroxy-steroid. These compounds have antioxidant and antimicrobial properties that can be widely used in the food industry.

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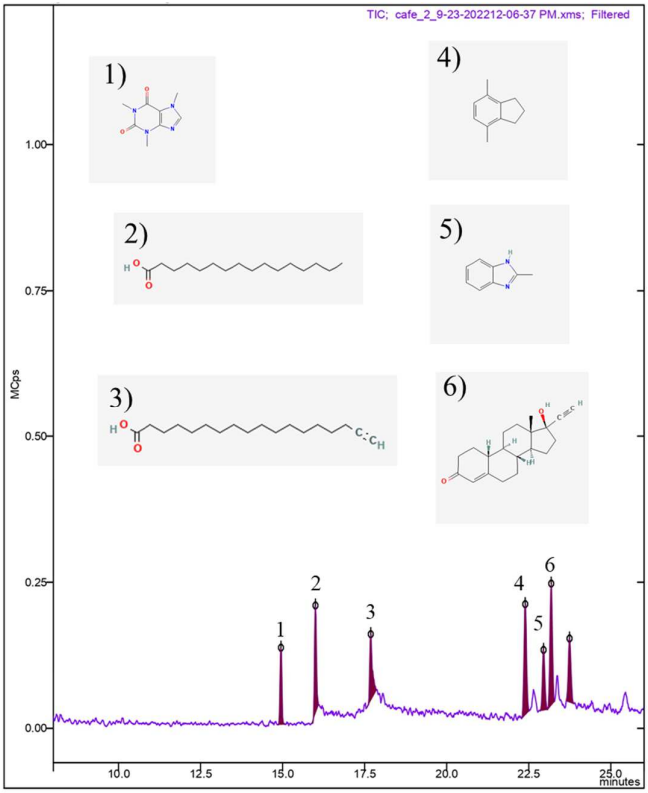

Figure 1. Gas chromatogram and major compounds of green coffee bean oil: 1) Caffeine, 2) n-Hexadecanoic acid, 3) 17-Octadecynoic acid, 4) 1H-Indene, 2,3-dihydro-4,7-dimethyl-, 5) Benzimidazole, 2-methyl-1-(3-phenylpropylthio)methyl-, and 6) Norethindrone. Database consulted: PubChem.

Table 1. Compounds identified by GC-MS in green coffee bean oil.

Peak n°	RT	Compound	% abundance	CAS
1	14.94	Caffeine	10.159	58-08-2
2	16.00	n-Hexadecanoic acid	14.344	57-10-3
3	17.64	17-Octadecynoic acid	12.244	34450-18-5
4	22.40	1H-Indene, 2,3-dihydro-4,7-dimethyl-	19.759	6682-71-9
5	22.96	Benzimidazole, 2-methyl-1-(3-phenylpropylthio)methyl-	10.540	615-15-6
6	23.19	Norethindrone	20.9440	68-22-4

3.2. High-performance liquid chromatography (HPLC) analysis

A total of 21 major compounds were identified in CP. Most of the identified compounds were phenolic compounds, terpenes and lignans (Figure 2, Table 2), such as the anthocyanins Delphinidin 3-O-galactoside and Delphinidin 3-O-arabinoside, the flavonols Quercetin 3-O-acetyl-rhamnoside and kaempferol, a biflavonid Theaflavin, diterpenoid andrographolide, isoflavone Biochanin A, secoiridoid glycoside Ligstroside, stilbenoid Resveratrol 3-O-glucoside, phenols and alcohol p-HPEA-EDA, lignans Secoisolariciresinol-sesquilignan and Cyclolariciresinol, disaccharide derivative Isorhamnetin 3-O-rutinoside, trihydroxyflavone Luteolin 7-O-glucuronide, glycosidal flavonoid Dihydromyricetin 3-O-rhamnoside and sesquiterpene lactone Artemisinin (database consulted: PubChem).

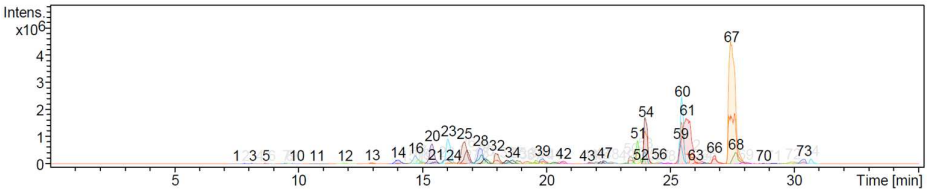

Figure 2. HPLC chromatogram and major compounds of coffee parchment.

Table 2. Chemical composition of coffee parchment, identified by HPLC.

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Peak n°	RT	Compound	Area	CAS	Formula
8	9.6	Delphinidin 3-O-galactoside	96352	197250-28-5	$C_{21}H_{21}O_{12}$
10	9.9	Delphinidin 3-O-arabinoside	115798	28500-01-8	$C_{20}H_{19}O_{11}$
11	10.7	Kaempferol 3-O-rhamnoside	216143	482-39-3	$C_{21}H_{19}O_{10}$
12	11.9	Isorhamnetin 3-O-galactoside	397750	5041-82-7	$C_{22}H_{22}O_{12}$
13	13	Quercetin 3-O-acetyl-rhamnoside	675166	Not available	$C_{23}H_{22}O_{12}$
14	14	Theaflavin	2433133	4670-05-7	$C_{29}H_{24}O_{12}$
15	15.0	Andrographolide	1412321	5508-58-7	$C_{20}H_{30}O_5$
20	15.4	Kaempferol	9936525	520-18-3	$C_{15}H_{10}O_6$
24	16.3	Kaempferol 3-O-(6"-acetyl-galactoside) 7-O-rhamnoside	658288	124097-45-6	$C_{29}H_{32}O_{16}$
28	17.3	Malvidin 3-O-(6"-p-coumaroyl-glucoside)	9548757	158189-28-7	$C_{32}H_{31}O_{14}$
33	18.4	Biochanin A	1985709	491-80-5	$C_{16}H_{12}O_5$
37	19.5	Ligstroside	958156	35897-92-8	$C_{25}H_{32}O_{12}$
42	20.7	3-Hydroxyphloretin 2'-O-xylosyl-glucoside	1290713	Not available	$C_{26}H_{32}O_{15}$
48	22.6	Resveratrol 3-O-glucoside	1275343	38963-95-0	$C_{20}H_{22}O_8$
51	23.7	p-HPEA-EDA	8929194	151194-92-2	$C_{17}H_{20}O_5$
55	24.4	Secoisolariciresinol-sesquilignan	446635	Not available	$C_{30}H_{38}O_{10}$
56	24.5	Isorhamnetin 3-O-rutinoside	801069	Not available	$C_{22}H_{22}O_{11}$
57	24.6	Luteolin 7-O-glucuronide	462237	29741-10-4	$C_{21}H_{18}O_{12}$
59	25.4	2-[4-(Diethylamino)-2-hydroxybenzoyl]benzoic acid	8324925	5809-23-4	$C_{18}H_{19}NO_4$
60	25.5	2-S-Glutathionyl caftaric acid	21393122	Not available	$C_{23}H_{27}N_3O_{15}S$
61	25.7	Dehydroeburicoic acid	48266948	6879-05-6	$C_{31}H_{48}O_3$
61	25.9	Dihydromyricetin 3-O-rhamnoside	4582788	Not available	$C_{21}H_{22}O_{12}$
64	26.4	Cyclolariciresinol	390934	548-29-8	$C_{20}H_{24}O_6$
71	29.2	Artemisinin	500292	63968-64-9	$C_{15}H_{22}O_5$

3.3. Elemental analysis of coffee parchment

The morphology of the ground and sifted CP is observed in microscopies, with heterogeneous sizes and shapes, obtaining elongated shapes larger than 20 µm (Figure 3).

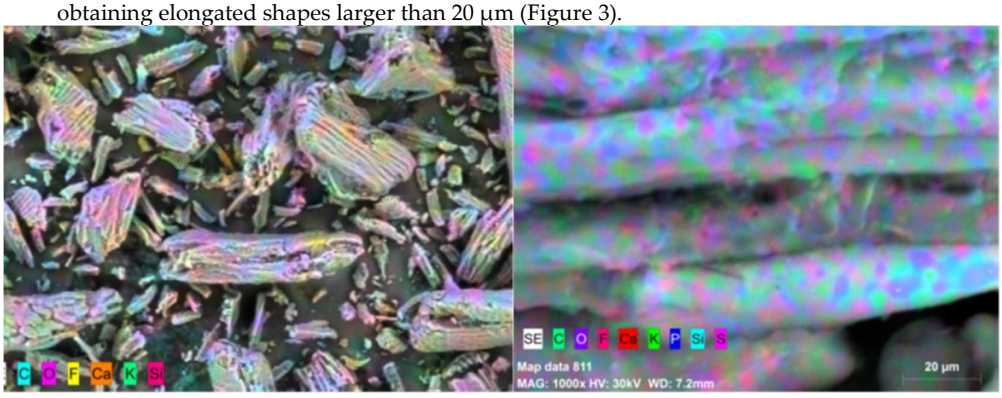

Figure 3. Environmental scanning electron microscopy (ESEM-EDS).

In the elemental analysis by EDS, oxygen and carbon were observed as major elements within CP (42 and 57% respectively) and 1% corresponds to minor elements such as calcium (0.08%) silicon (0.05%), sulfur (0.02%), calcium (0.13%), iron (0.13), potassium (0.01) and fluorine (0.11%). This study confirms that the CP sample did not contain organic pollutants within its composition (Figure 4).

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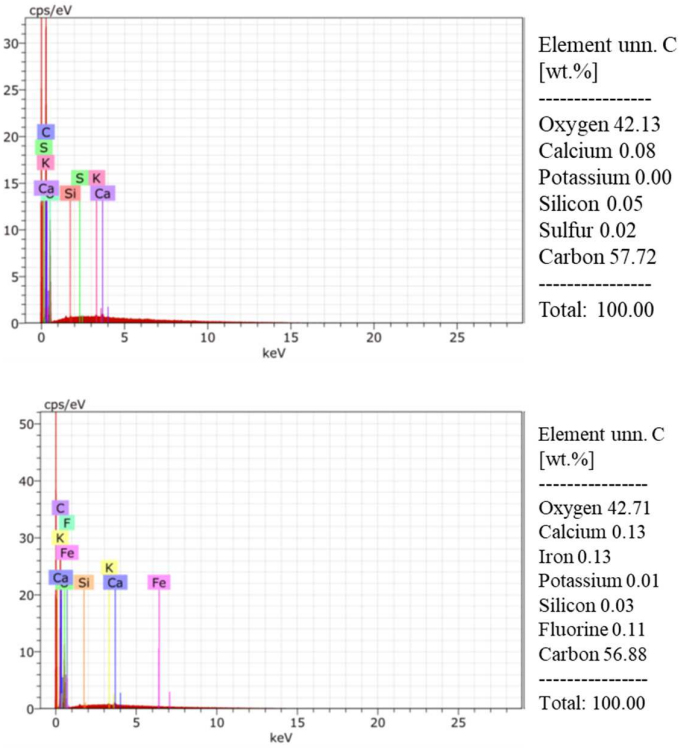

Figure 4. Spectra of the EDS elemental analysis of the coffee parchment.

3.4. In vitro effect of coffee residues, chitosan and nanoparticulas de chitosan on mycelial growth and spore germination inhibition of Botrytis sp. and Rhizopus sp.

In the case of Botrytis sp., a mycelial growth rate (MGR) of 7.25 mm/day was observed in the control (PDA), similar to the treatments of CP 1% and CP 5% with a MGR of 7.19 and 6.55 mm/day respectively. These treatments with CP presented a stimulatory effect on mycelial growth, with exception of CP at 10% the inhibitory effect was (51.98 %). In contrast to GCBO in the three concentrations, they presented a 100% inhibition. Additionally, the treatments with NC, Ch and ChNp at concentrations of 10 and 20% showed a greater inhibitory effect (100%), as shown in Table 3 and Figure 5A. Most treatments inhibited the formation spores with exception of the 1% CP, 1% NC, 1% Ch and 1% ChNp treatments presented higher percentages of SGI compared to the control; only the CP 5% treatment presented a slightly positive percentage of germination inhibition of Botrytis spores compared to the control.

Table 3. In vitro antifungal effect of coffee residues, chitosan and chitosan nanoparticles on *Botrytis* sp.

Tratment	Grow rate (mm/day)	Mycelial growth on the last day	Inhibition of mycelial growth (%)	Inhibition of spore germination (%)
Control	7.25	50±0 d	0 a	0
CP 1%	7.19	48.14±1.11 d	3.72 bc	-7.98
CP 5%	6.55	44.31±4.22 c	11.38 c	4.19
CP 10%	3.16	24.01±3.42 b	51.98 d	NSF
GCBO 1%	NG	0 a	100 f	NSF
GCBO 3%	NG	0 a	100 f	NSF
GCBO 6%	NG	0 a	100 f	NSF
NC 1%	7.09	47.54±1.20 d	4.92 bc	-3.39
NC 10%	NG	0 a	100 f	NSF
NC 20%	NG	0 a	100 f	NSF
Ch 1%	7.37	49.43±0.45 d	1.14 ab	-3.39
Ch 10%	NG	0 a	100 f	NSF
Ch 20%	NG	0 a	100 f	NSF
ChNp 1%	7.18	48.08±1.640 d	3.84 b	-8.98

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ChNp 10%	NG	0 a	100 f	NSF
ChNp 20%	NG	0 a	100 f	NSF

CP = coffee parchment; GCBO = green coffee bean oil; NC = nanostructured coating; Ch = chitosan solution; ChNp = chitosan nanoparticles. One-way anova, Tukey α=0.05; F= 2164.85; gl =74; standard error = 1.4207; p<0.0001. Different letters indicate significant differences, NSF= no spore formation, NG= no growth.

In Rhizopus sp., a MGR of 1.26 mm/hour was observed in the control (PDA), while in the CP at the different concentrations, a higher rate was observed due to the stimulation of mycelial growth. In the case of the oil, a decrease in the MGR was observed (0.66 for 1%, 0.54 for 3% and 0.83 for 6%) and MGI of 43.38 to 31.5%. For NC and Ch at the highest dose of 20% the inhibition mycelial growth was 100%; however, the 1% dose presented a stimulation with a MGR of 1.28 mm/hour. In ChNp, the lowest dose of 1% presented a MGR of 1.24 mm/hour, but the doses of 10 and 20% did not show mycelial growth. These data can be compared with the inhibition percentages shown in Table 4 and Figure 5B. The treatments that presented a 100% inhibition of the mycelial growth of Rhizopus sp. were 20% NC, 20% Ch and 10 and 20% ChNp while treatments with CP did not inhibit mycelial growth. Coffee oil and chitosan nanoparticles at all concentrations inhibited spore formation. The percent inhibition of spore germination presented values from 1.92% to 17.86% and present significant differences (p<0.0001) between the treatments of CP, NC and Ch. In the germination inhibition of Rhizopus spores, the CP treatments presented the highest percentages of SGI together with 20% Ch; However, all treatments with GCBO and ChNp did not present spore formation. High concentrations prevent mycelium development, but spores were not formed at low concentrations despite the presence of mycelium.

Table 4. In vitro antifungal effect of coffee residues, chitosan and chitosan nanoparticles on *Rhizopus* sp.

Tratment	**Grow rate (mm/day)**	**Mycelial growth on the last day**	**Inhibition of mycelial growth (%)**	**Inhibition of spore germination (%)**
Control	1.26	50±0 d	0 a	0 a
CP 1%	1.28	50±0 d	0 a	12.93 ef
CP 5%	1.28	50±0 d	0 a	17.86 ef
CP 10%	1.28	50±0 d	0 a	16.25 f
GCBO 1%	0.66	28.31±7.41 b	43.38 de	NSF
GCBO 3%	0.54	24.20±2.01 b	51.6 e	NSF
GCBO 6%	0.83	34.25±1.17 c	31.5 d	NSF
NC 1%	1.24	48.55±1.19 d	2.9 b	4.72 bcd
NC 10%	0.54	24.14±6.03 b	51.72 e	1.92 b
NC 20%	NG	0 a	100 f	NSP
Ch 1%	1.28	50±0 d	0 a	8.21 cde
Ch 10%	1.14	45.02±0.69 d	9.96 c	3.32 def
Ch 20%	NG	0 a	100 f	14.51 bc
ChNp 1%	1.24	48.63±1.28 d	2.74 b	NSF
ChNp 10%	NG	0 a	100 f	NSF
ChNp 20%	NG	0 a	100 f	NSF

CP = coffee parchment; GCBO = green coffee bean oil; NC = nanostructured coating; Ch = chitosan solution; ChNp = chitosan nanoparticles. One-way anova, Tukey α=0.05; F=379.76; gl =74; standar error = 6.3158; p<0.0001. Different letters indicate significant differences. NSF= no spore formation, NG= no growth.

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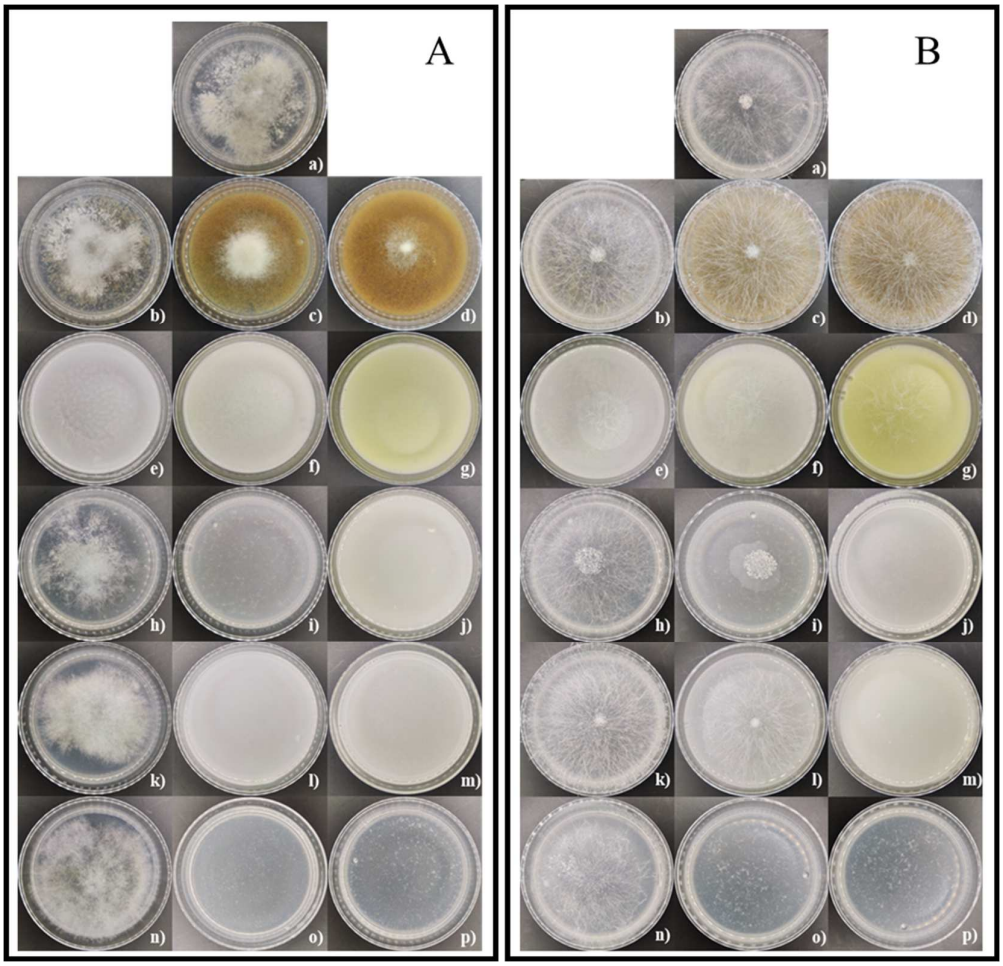

Figure 5. In vitro assay on mycelial growth. A) *Botrytis* sp. B) *Rhizopus* sp. a) Control (PDA), b) 1% CP, c) 5% CP, d) 10% CP, e) 1% GCBO, f) 3% GCBO, g) 6% GCBO, h) 1% NC, i) 10% NC, j) 20% NC, k) 1% Ch, l) 10% Ch, m) 20% Ch, n) 1% ChNp, o) 10% ChNp, p) 20% ChNp.

3.5. In vitro assay of coffee residues and incubation times on mycelial growth and spore germination inhibition of Botrytis sp. and Rhizopus sp.

There were no differences in mycelial growth of Botrytis sp. between incubation times or between treatments at different concentrations. The 10% CP treatment showed an initial growth of 5 mm to 24 mm at 8 days compared to the 50 mm control (Figure 6a). In Rhizopus sp., the 1% GCBO, 3% GCBO, 6% GCBO, 10% NC and 10% CP treatments showed the lowest mycelial growth with significant differences compared to the control ($p<0.001$). (Figure 6b).

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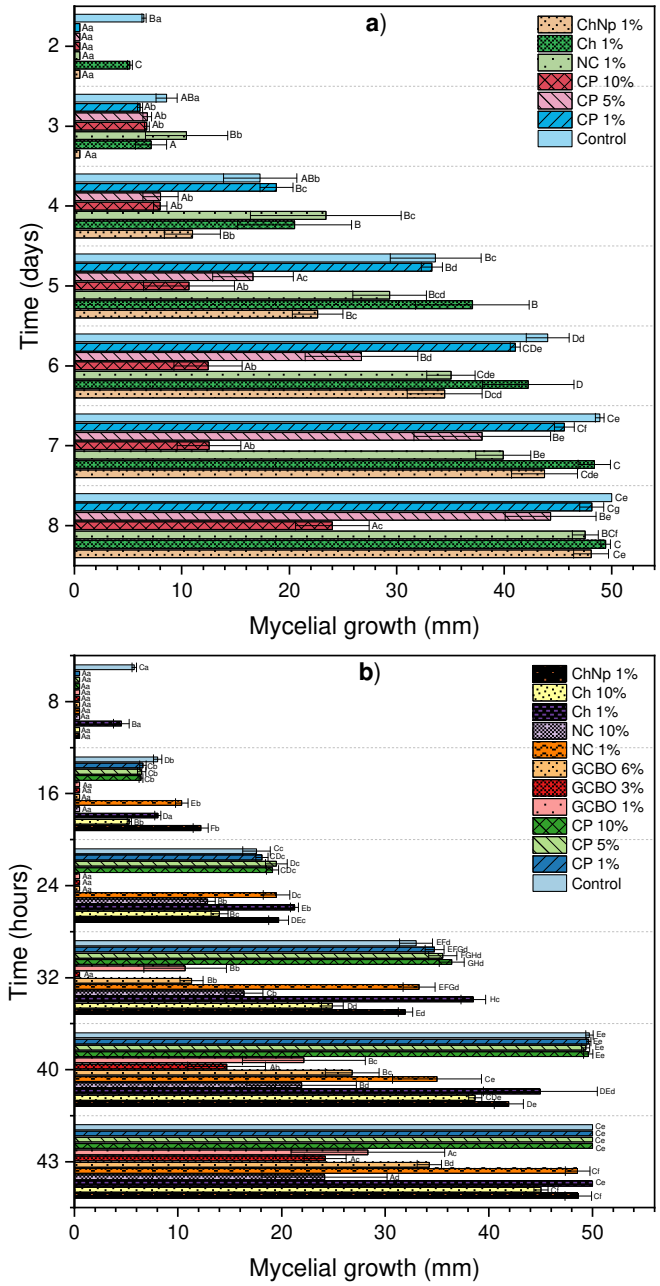

Figure 6. In vitro mycelial growth inhibition with respect to time, capital letters represent significant differences between treatments and lowercase letters represent differences with respect to time. a) *Botrytis* sp. comparison of Tukey means (means ± SD), α = 0.05; DMS=2.22794; gl = 194; standard error= 9.1862; p< 0.0001. b) *Rhizopus* sp. with Tukey's comparison of means (means ± SD), α = 0.05; DMS=2.09301; gl = 266; standard error= 4.9471; p< 0.0001.

In the inhibition of spore germination of Botrytis sp. and Rhizopus sp. with respect to time, no significant differences were observed (p < 0.001) between the treatments, except for the treatment with 10% NC at 4 and 6 hours on Rhizopus sp., as shown in Figure 7.

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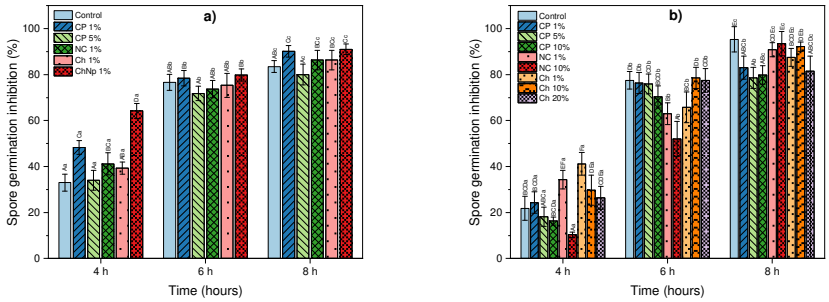

Figure 7. Inhibition of spore germination. a) *Botrytis* sp. One-way ANOVA with Tukey comparison of means (means ± SD) α= 0.05; F= 143.864, p<0.001. b) *Rhizopus* sp. One-way ANOVA with Tukey comparison of means (means ± SD) α= 0.05; F= 252.163, p<0.001. Bars represent the standard error of the mean and Different letters show a significant difference between the same treatments over time.

3.6. In vivo effect

Variations in incidence and severity were observed in blueberry fruits. The in vivo test for Botrytis sp. showed the lowest incidence after 10 days of evaluation (40%) with the commercial fungicide, it was more effective than the other treatments and the control (66.66%). The CP and NC treatments presented an incidence of 46.66%. In terms of severity, the control treatment showed the highest values with 34%, while the NC treatments (17.73%) and CP (16.66%) were more effective in reducing fruit damage (Fig 8, Fig 10-a1, 10-a2).

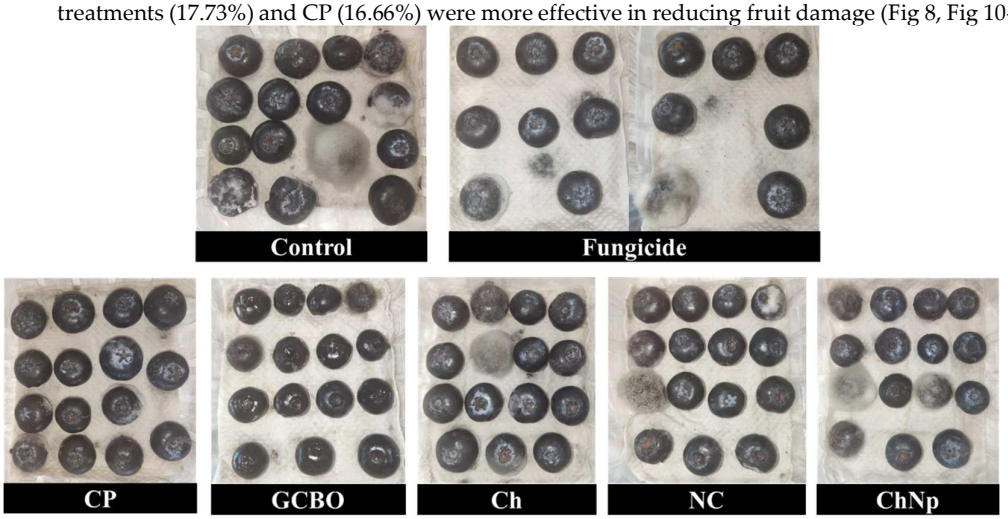

Figure 8. Incidence and severity of *Botrytis* sp. on blueberry.

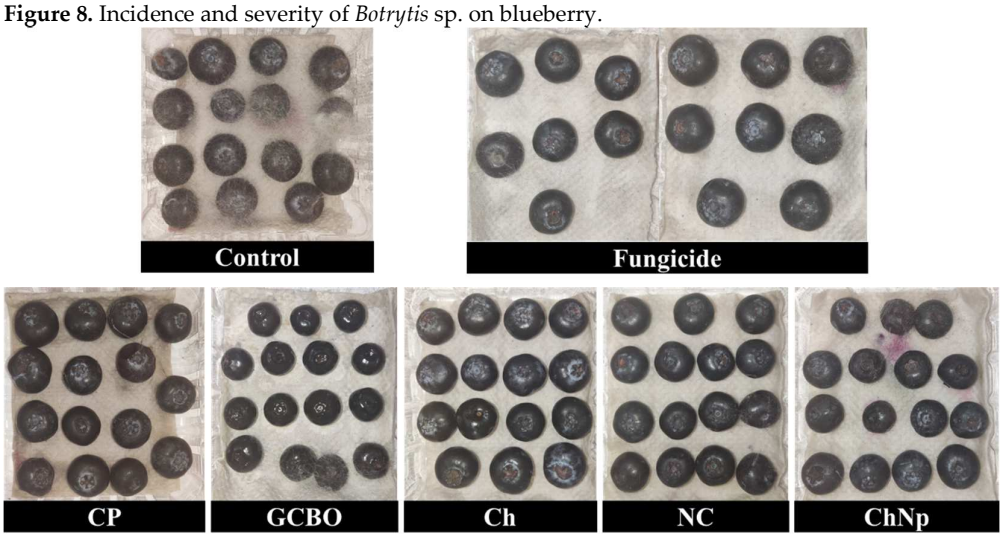

Figure 9. Incidence and severity of *Rhizopus* sp. on blueberry.

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For Rhizopus sp., the in vivo test was 4 days (Figure 9, Fig 10-b1, 10-b2). The incidence in the control was the most susceptible with (100%), while the lowest incidence was observed in Ch treatment (13.33 %). The severity in the control group reached 100% while the Ch treatment was the most effective with a severity of 0.86%. All treatments had the lowest incidence and severity on blueberry.

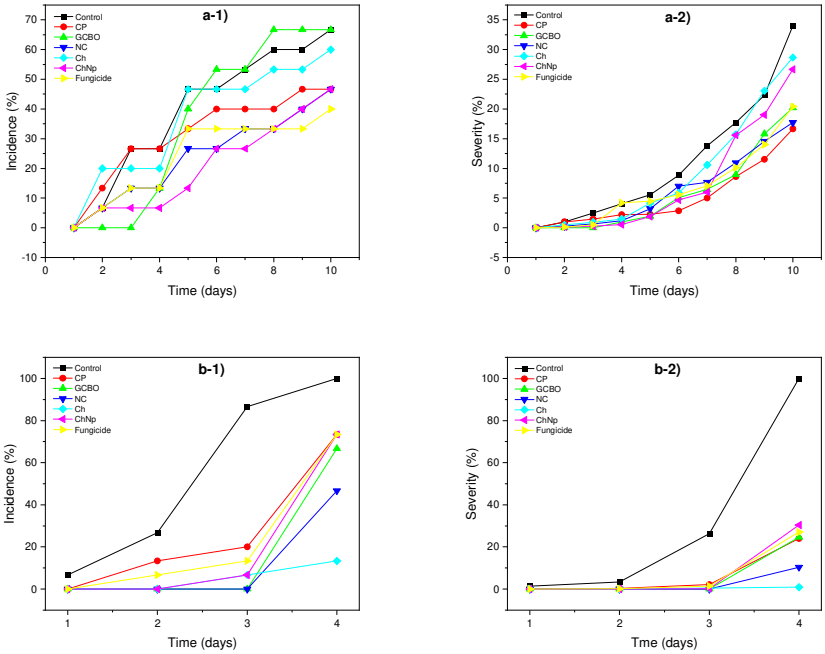

Figure 10. Effect of coffee residues and chitosan on the incidence (1) and severity (2) of blueberry fruits inoculated with a) *Botrytis* sp. and b) *Rhizopus* sp.

3.7. In vitro assay of coffee residues incorporated into the PM

The results are shown in Table 5. A fungistatic effect was obtained in all treatments, with significant differences ($p<0.0001$). The PP+NC treatment was the most effective with a mycelial growth inhibition of 63.04%, while the least effective was PET with an inhibition of 11.68% on Botrytis sp. compared to the control. In Rhizopus sp., a fungistatic effect was also obtained. Compared to the control, mycelial growth was inhibited 14.28% with PET treatments and 15.78% with PPCP treatments. The PPCP+NC, PP, PP+NC treatments showed 100% inhibition mycelial growth during the time evaluated. However, they presented a fungistatic effect; the fungus continued its development after replanting in PDA medium.

Table 5. In vitro effect of PM on Botrytis sp. and Rhizopus sp.

	***Botrytis* sp.**		***Rhizopus* sp.**	
Tratment	**Mycelial growth on the last day (mm)**	**Inhibition of mycelial growth (%)**	**Mycelial growth on the last day (mm)**	**Inhibition of mycelial growth (%)**
Control	50±0	0 a	50±0 b	0 a
PET	44.16±16	11.68 b	42.86±3.68 b	14.28 b
PPCP	40.61±2.08	18.78 b	42.11±8.09 b	15.78 b
PPCP+NC	40.60±4.99	18.8 b	0 a	100 c
PP	27.98±2.62	44.04 c	0 a	100 c
PP+NC	18.48±5.87	63.04 d	0 a	100 c

Control=PDA, PET=Polyethylene Terephthalate, PPCP=PLA/PBAT with CP without NC, PPCP+NC=PLA/PBAT with CP and NC, PP=PLA/PBAT without NC and PP+NC=PLA/PBAT with NC.

For mycelial growth with respect to time, significant statistical differences were obtained ($p<0.0001$), as shown in Figure 11. Lineal mycelial growth was observed in all treatments against *Botrytis* sp. The lowest mycelial growth occurred with PP and PP+NC treatments (Figure 11a). For *Rhizopus* sp., significant differences in mycelial growth were observed with respect to time. The control treatment showed mycelial growth at 10

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h, and the PET treatment showed growth at 15 h. The PPCP+NC treatment showed growth at 25 h. No mycelial growth was observed in PPCP, PP and PP+NC treatments at the evaluated times (Figure 11b).

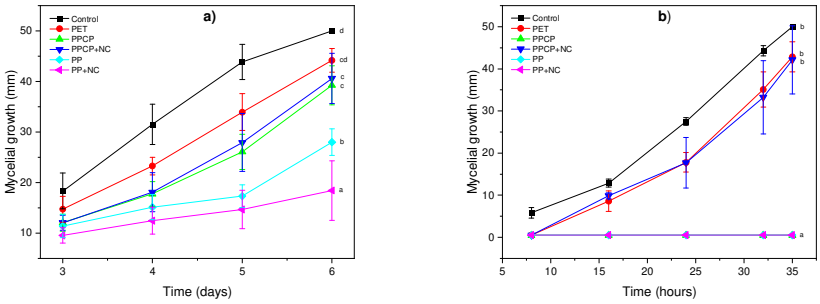

Figure 11. Effect of the PM on the inhibition of mycelial growth. Control=PDA, PET=Polyethylene Terephthalate, PPCP=PLA/PBAT with CP without NC, PPCP+NC=PLA/PBAT with CP and NC, PP=PLA/PBAT without NC and PP+NC=PLA/PBAT with NC. a) *Botrytis* sp. comparison of Tukey means (means ± SD), $\alpha = 0.05$; DMS=7.05354; gl = 24; standard error= 12.4901; p< 0.0001. Bars represent the standard error of the mean. b) *Rhizopus* sp. with Tukey's comparison of means (means ± SD), $\alpha = 0.05$; DMS=7.95035; gl = 17; standard error= 10.9018; p<0.0001. Bars represent the standard error of the mean.

4. Discussion

Regarding the content of volatiles present in green coffee bean oil, caffeine is a typical compound of the coffee plant characterized by having a high antioxidant activity [29]; however, caffeine is also characterized by having a broad antimicrobial activity. It is present in various parts of the coffee plant. Nonthakaew et al. [30] evaluated the antimicrobial activity of caffeine extracts from coffee beans on the mycelial development of *Rhizopus oryzae* at concentrations of 2.5 to 10 g 100 mL^{-1}. This occurs because caffeine can inhibit fungi by stopping the synthesis of glucose, fructose and maltose, preventing fungal spread. Other compounds such as hexadecanoic acid and octadecinoic acid with antimicrobial activity have been identified in coffee [31]. Phenolic compounds are abundant in coffee by-products, since they are characterized by their antimicrobial activity [32]. These are abundant in the CP and have the function of covering and protecting the bean from attacks by other microorganisms.

Kaempferol has been reported as a bioactive compound characterized by its high antioxidant activity and is present in various parts of the coffee plant [33]. We found kaempferol 3-O-rhamnoside present at retention time 10.7, kaempferol at retention time 15.4, kaempferol 3-O-(6''-acetyl-galactoside) 7-O-rhamnoside at retention time 16.3 due to its wide distribution in this residue, acting as defense substances in coffee with antioxidant activity. Periferakis et al. [34], found that kaempferol was a flavonoid widely distributed in several plant species and acting as an antimicrobial agent. The antifungal activity of kaempferol can be enhanced if it is encapsulated in chitosan nanoparticles, as mentioned by IlK et al. [35], who evaluated the antifungal effect of kaempferol and kaempferol loaded lecithin/chitosan nanoparticles on *Fusarium oxysporium*. Using kaempferol alone resulted in 100% growth on day 20 of evaluation, while using encapsulated kaempferol resulted in a growth greater than 60% on day 60. Quercetin-3-O-galactoside and isorhamnetin inhibit bacterial division and growth during the logarithmic growth period and acts as a bacterial inhibitor. Inhibition may be mediated by the disruption of the bacterial cell structure, leading to leakage of contents including sugars, nucleic acids, and proteins [36]. The antibacterial activity of theaflavin: epicatechin combinations against Candida albicans was stronger than that of theaflavin alone. Minimum inhibitory concentrations (MICs) of 1024 μg/ml with theaflavin and 128-256 μg/ml with theaflavin:epicatechin combinations were observed by Betts et al. [37]. The compound Andrographolide has anti-inflammatory and antioxidant effects; however, it also has properties for the control of infections caused by microorganisms, due to the capacity of intracellular DNA inhibition [38,39]. In Grape pomace was isolated 2-S-Glutathionyl caftaric acid and has been reported with antimicrobial activity on three bacterial strains [40]. *Ficus eriobotryoides* leaves were reported with antifungal activity on six isolates of *Fusarium oxysporium* and contained Myricetin [41]. Although the CP has a wide content of compounds, these mostly act as antioxidants, which is why CP has the lowest antifungal effects compared to other

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treatments. Mirón-Mérida et al. [9] evaluated ethanolic extracts of CP on the mycelial growth of *Colletotrichum gloesporioides* and *Fusarium verticillioides* and found greater inhibition as the extract dose is increased; this is due to the presence of bioactive compounds, most of which are phenolic compounds. In our research the most sensitive fungus to CP was *Botrytis* sp. at a dose of 10% which can be attributed to the presence of the major phenolic components identified by the HPLC-MS analysis.

In the elemental analysis of CP, values of 57.72 and 56.88% of carbon were obtained, coinciding with results from Coura et al. [42], who reported a carbon content of 60.5 and 62.3% in CP because it is a lignocellulosic material rich in carbon and hydrogen. It has the capacity to release energy due to its high combustion enthalpy and this means that CP could absorb organic compounds on its surface [43].

Several bioactive antifungal compounds have been identified in CP. In the in vitro tests, each treatment presented differing effects due to the differences in its bioactive constituents, but green coffee bean oil, showed total inhibition in formation of spores in both fungi due to its volatile phase, which prevents its evaporation when in a closed medium such as the Petri dish. The lipophilic phase of the oil is more easily absorbed by the mycelium [44].

Chitosan has physicochemical properties that provide it with an antifungal effect. It can disintegrate the cell wall membrane of fungi, cause cytoplasm leakage, chelation of essential nutrients and the binding of nucleic acids that alter the flow of genetic information [45]. By synthesizing chitosan as a nanoparticle, significant inhibitory effects are obtained, with changes occurring in colony formation and spore germination, and nanoparticles induce morphological changes by having a more effective membrane penetration [46]. El-Naggar et al. [47], compared the effect of chitosan and chitosan nanoparticles in inhibiting *Botrytis cinerea* mycelium, and showed greater inhibition when using nanoparticles. At a concentration of 1 mg/mL with chitosan, they obtained an inhibition of 31.16%, while they obtained an inhibition of 73.58% when using chitosan nanoparticles at the same concentration. This may be explained by the alteration in membrane permeability due to morphological changes that inhibit mycelial growth, sporulation and spore germination.

In the in vivo test, blueberry fruits contain a high content of phenolic compounds and anthocyanins, which function to preserve the fruits. One way to avoid the loss of these compounds is the application of coatings such as essential oils or nanoemulsions that help preserve these compounds and protect the fruits against fungal attacks [48]. The CP and NC treatments presented minor incidence and severity on *Botrytis* sp. and Ch treatments on *Rhizopus* sp. The CP contain a high content of phenolic compounds and NC or Ch have solution chitosan and chitosan nanoparticles which may be responsible for this biological activity in vivo. Sun et al. [49] evaluated a coating of chitosan and carvacrol essential oil and found that the inhibitory effect against *Escherichia coli* and *Penicillium digitatum* was greater when using the treatments together than separately. Therefore, mixing a bioactive oil with chitosan can enhance its antimicrobial properties.

To evaluate the PM, the addition of active compounds in polymeric materials can serve as a controlled release vehicle against microorganisms as reported by Black-Solis et al. [50], who evaluated the addition of cinnamon essential oil and chitosan solution to PLA and PBAT polymeric fibers. They were tested against *Alternaria alternata* and inhibited its mycelial growth and spore germination at doses of 6.1% cinnamon essential oil.

5. Conclusion

The in vitro effect of coffee residues occurred mainly on the mycelial growth of *Botrytis* sp. with 100% inhibition in the presence of GCBO, NC, Ch and NpCh, while in *Rhizopus* sp. 20% NC, Ch and NpCh obtained 100% inhibition. For the inhibition of spore germination, GCBO was the best treatment, since it prevented spore growth at all doses. The incidence and severity in the fruits was 16 and 17% in blueberries treated with CP and NC stored at room temperature. In the in vitro evaluation the PM showed a significant fungistatic effect on both fungi. In this work, the inhibitory effect on *Botrytis* sp. and *Rhizopus* sp. in vitro and in vivo was enhanced by incorporating coffee waste into a polymeric matrix, which could be used to produce biodegradable packaging to extend the shelf life of fruit and vegetable. The PM can be an alternative for the production of active packaging since they are made with different natural components CP, NC and GCBO whose purpose is to release the compounds within the food packaging.

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

Abbreviations

The following abbreviations are used in this manuscript:

PLA	Polylactic acid
PBAT	Poly(butylene adipate-co-terephthalate)
CP	Coffee parchment
GCBO	Green coffee bean oil
Ch	Chitosan solution
ChNp	Chitosan nanoparticles
PM	Polymer matrix
PM+CW	Polymer matrix with coffee waste
TLA	Three letter acronym
NC	Nanostructured coating
PPCP	Polylactic acid/Poly(butylene adipate-co-terephthalate)+ Coffee parchment
PP+NC	Polylactic acid/Poly(butylene adipate-co-terephthalate)+ Nanostructured coating
PPCP+NC	Polylactic acid/Poly(butylene adipate-co-terephthalate)+ Coffee parchment+ Nanostructured coating
GC-MS	Gas chromatography and mass spectrometry
HPLC-MS	Liquid chromatography and mass spectrometry
ESEM-EDS	Environmental scanning electron microscopy
MI	Mycelial inhibition
Gr	Growth rate
DF	Diameter of the final growth
DI	Diameter of the initial growth
SGI	Spore germination inhibition
MGR	Mycelial growth rate
NG	No growth
NSF	No spore germination

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