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# Antimicrobial activity of coffee bagasse (Coffea arabica) extract against phytopathogenic bacteria and fungi

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# Abstract

Plant diseases caused by microbial pathogens represent a major challenge for global agriculture, leading to annual crop losses estimated at more than USD 220 billion. The search for sustainable antimicrobial agents derived from agro-industrial by-products has gained increasing attention as an alternative to synthetic pesticides. Coffee bagasse, a major residue generated during coffee processing, retains a wide range of bioactive compounds with potential antimicrobial properties. This study aimed to evaluate the antibacterial and antifungal potential of an ethanolic extract of coffee bagasse against opportunistic strains relevant to agriculture. Chemical characterization (total phenols, total melanoidins, target analysis of phenolic compounds, caffeine, amino acids, and sugars) revealed main chemical compounds such as chlorogenic acid, gallic acid, caffeine, and melanoidins. The most abundant amino acids were serine, gamma-aminobutyric acid (GABA), alanine, and aspartic acid. Antimicrobial assays showed promising activity against phytopathogenic bacteria *Erwinia amylovora* and *Dickeya dadantii*. Fungi such as *Alternaria alternata, Fusarium oxysporum*, and *Penicillium charlesii* exhibited concentration-dependent susceptibility, with optimal inhibition at 10 mg/mL. These findings demonstrate the potential revalorization of coffee bagasse as a sustainable source of antimicrobial compounds with possible applications in plant disease management and food safety.


# Introduction

According to the Food and Agriculture Organization of the United Nations (FAO), plant diseases cause an estimated global loss of up to 220 billion USD annually, and the conventional treatments used to control these diseases are often insufficient or ineffective [1, 2]. Plant pathogens are mostly certain microorganisms such as certain bacteria, viruses, fungi, nematodes, and protozoa that can attack plants causing plant diseases, which cause disease in humans and animals (Table S1) [3]. Different approaches have been applied to mitigate biotic stress, chemical control being an effective management option for many plant pests. However, the development of resistance against chemical pesticides and the large impact of their residues on human health and the environment have prompted the search for novel eco-friendly strategies to ensure crop health and production [4, 5].

On the other hand, coffee is considered one of the most popular beverages among consumers. Globally, it is valued for its flavor, neurostimulant effect, and its role in social interaction, often regarded as a recreational activity. The effort to meet the global demand for coffee has led to its production in enormous quantities. By the 2020–2021 period, global coffee production reached nearly 11 million tons of roasted coffee beans. This massive production has generated a significant amount of waste at various stages of the coffee processing and production chain. These residues amount to more than two billion tons annually [6].

One of the main by-products of the coffee industry is coffee bagasse, also known as spent coffee grounds. Approximately 6 million tons of this waste are generated each year. It is an organic residue composed of ground and roasted coffee beans, obtained during the coffee brewing process after the

beverages are prepared. Coffee bagasse represents about 90% of the original mass of the coffee beans, meaning only 10% of the beans are converted into the actual beverage [7, 8, 9]. However, this agro-industrial waste retains many of the phytochemical components from the original coffee seeds, including bioactive compounds that have already demonstrated significant biological activity, such as coffee antioxidant, anti-inflammatory, and antimicrobial properties, among others [6, 7, 10].

Notably, it has been found that coffee bagasse contains bioactive compounds with antimicrobial activity, particularly phenolic acids such as chlorogenic acid, caffeic acid, gallic acid, and ferulic acid, among others [11, 12]. Previous studies have already demonstrated the antimicrobial potential of coffee and its derivates, demonstrating efficacy against various Gram-positive and Gram-negative pathogens of healthcare and nutritional relevance [10]. However, their applications have mostly been limited to the clinical and food sectors, leaving aside other areas that could also benefit from these properties [13, 14, 15].

Due to coffee bagasse retaining a significant number of antimicrobial compounds, which are originally present in the beans, we hypothesize that it could be repurposed in the agricultural sector for treatment or prevention of crop diseases and the loss of marketability of crops contaminated by non-phytopathogenic bacteria that cause human and animal health problems. Therefore, the present study aimed to characterize the bioactive composition of coffee bagasse and evaluate the antibacterial and antifungal activity of its ethanolic extract against phytopathogens responsible for diseases in agriculturally important crops and bacteria that cause foodborne illness and in consequence reduce crop commercialization (*Supplementary Material Table S1*).

## Results

# Chemical characterization of ethanolic extract of dry coffee bagasse

The spectrophotometric quantification of total phenols in the ethanolic extract of coffee bagasse yielded a value of 91.26 mg of gallic acid equivalents per gram of sample (mg GAE/g).

Eight bioactive compounds were identified in the ethanolic extract of coffee bagasse, seven of which correspond to phenolic acids and one to an alkaloid. The identified phenolic compounds were: caffeic acid, chlorogenic acid, ferulic acid, sinapic acid, *p*-coumaric acid, gallic acid, and syringic acid, while the detected alkaloid was caffeine. The three most abundant compounds were gallic acid, caffeine, and chlorogenic acid, with concentrations of 85.58 mg/g, 47.65 mg/g, and 15.31 mg/g, respectively (Table 1).

The ethanolic extract of coffee bagasse showed a value of 14.96 g of total melanoidins per 100 g of dry coffee bagasse extract.

In the sugar content analysis of the ethanolic extract of coffee bagasse, none of the reference carbohydrates used as standards, including glucose, sucrose, fructose, maltose, galactose, mannitol, trehalose, raffinose, and mannose, were detected.

The amino acid analysis of the coffee bagasse extract revealed the presence of 16 amino acids: aspartic acid, glutamic acid, serine, asparagine, glycine, histidine, threonine, alanine, gamma-aminobutyric acid (GABA), proline, tyrosine, valine, isoleucine, leucine, lysine, and phenylalanine. Among these, the most abundant were serine, gamma-aminobutyric acid (GABA), alanine, and aspartic acid (34.11± 0.51, 14.51 ± 3.17, 9.72 ± 0.81, 9.68 ± 0.51 pmol/mg bagasse extract, respectively) (Table 2). The results are expressed in pmol of amino acids per mg of extract. The total amino acid content detected in the extract was 100.98 ± 8.24 pmol/mg of dry coffee bagasse extract.

## Antibacterial Activity of the Ethanolic Extract

The MIC of the ethanolic extract of coffee bagasse against *S. aureus* was 5 mg/mL, while the MBC was 8 mg/mL. No inhibitory effect was observed for chlorogenic acid within the tested concentration range. In the case of gallic acid, bacterial growth reduction began to appear at a concentration of 2 mg/mL; however, no complete inhibitory or bactericidal effect was observed (Fig. 1A).

For *E. coli*, the MIC was > 20 mg/mL, and no MBC was detected within the tested concentrations.

Chlorogenic acid showed no inhibitory or bactericidal effect, while gallic acid completely inhibited bacterial growth at a concentration of 5 mg/mL (Fig. 1B).

Growth curves of *E. amylovora* and *D*. *dadantii* over 24 hours, are reported (Fig. 2). The y-axis represents the optical density readings reported by the microplate reader, and the x-axis shows the elapsed time. In this context, lower optical density indicates lower bacterial growth. In Fig. 2A, the growth kinetics of *E. amylovora* are shown. The dark blue line represents the control, which indicates the normal growth behavior of the bacterium, followed by the treatment groups (extract concentrations). In this case, a deviation from the normal growth kinetics can be observed starting at a concentration of 5 mg/mL, compared to the control. However, the greatest effectiveness of the extract was observed at concentrations of 15 and 20 mg/mL, preventing further bacterial development since the beginning of the curve (0–18 h) compared to the control. In Fig. 2B, the growth kinetics of the phytopathogen *D. dadantii* are shown. The control line illustrates how this bacterium behaves under optimal growth conditions. In this case, concentrations of 10, 15, and 20 mg/mL exhibit a similar inhibitory effect on bacterial growth, which remains stable for at least 24 hours at the two highest concentrations. The effects of chlorogenic acid, gallic acid, and both at concentrations equivalents to of 15 and 20 mg/ml of extract was determined (Fig. 3). Figure 3 showed that for none of the phytopathogenic strains tested, the addition of chlorogenic acid, gallic acid, or both at concentrations equivalents to 15 and 20 mg/ml of extract, respectively, had any effect on the strains comparing to ethanolic extract of coffee bagasse,

demonstrating that the biocidal effect is attributed to the synergy of the compounds present in the ethanolic extract of coffee bagasse and not to the major components presented in it.

## Antifungal Activity of the Ethanolic Extract

Six phytopathogenic fungi were subjected to an initial antifungal activity assay: *A. alternata, P. aurantiogriseum, B. cinerea, F. oxysporum, P. digitatum*, and *P. charlesii* (Table S1). In the first experiment, the effect of the extract was evaluated at low concentrations: 0.2, 0.4, and 1 mg/mL. Only three of the tested fungi showed signs of susceptibility to the ethanolic extract of coffee bagasse (Fig. 3A); therefore, *A. alternata*, *F. oxysporum*, and *P. charlesii* were selected for further analysis.

For the subsequent biocidal analysis, concentrations of 1, 2, 3, 5, and 10 mg/mL were used. In all cases, a concentration-dependent inhibitory effect was observed, meaning that fungal growth decreased as the extract concentration increased. In Fig. 4A a bar graph shows the antifungal effect observed in the three fungi, including the concentrations from the two assays. The microorganisms that exhibited the highest susceptibility to the extract were *F. oxysporum* and *A. alternata*. As shown in Fig. 4A, the highest concentration tested (10 mg/mL) reduced fungal growth by half compared to the control. *P.charlesii*, although it did show susceptibility to the coffee bagasse extract, was the most resistant fungus compared to the other two.

Significant differences were found for the fungal species used, the concentration, and the interaction effect between species and concentration, with a P-value < 0.0001 in all three cases. Tukey's test ($P < 0.05$) revealed significant differences between the control and the rest of the treatments. The concentrations of 0.2 and 1 mg/mL were statistically equal, as were the concentrations of 0.4 and 2 mg/mL. The remaining treatments were statistically different. Likewise, the behavior among the fungal species was also significantly different ($P < 0.05$). However, the greatest differences are observed in the control compared to the concentrations of 3, 5 and 10 mg/mL of coffee bagasse extract. In Fig. 4B, fungal growth is shown at the highest extract concentrations tested (3, 5 and 10 mg/mL), as these were the ones that produced the best results.

In the following assay, the individual effects of the pure compounds gallic acid and chlorogenic acid were tested. For two of three fungi, the compound that exhibited the greatest biocidal effect was gallic acid, with an amount equivalent to 100 mg/mL of bagasse (8.858 mg/mL) proving lethal for *A. alternata* and *F. oxysporum* (Figure S1.A). In the case of *P. charlesii*, although its growth was also slightly compromised, the results were not as conclusive as those for the other fungi, as it was still able to develop, albeit to a lesser extent (Figure S1.A). In the case of chlorogenic acid, the biocidal effect observed with this compound alone was much milder than that of gallic acid at the same equivalent amount (1.53 mg/mL, corresponding to 100 mg of coffee bagasse extract) (Figure S1.B). However, the biocidal effect of *P. charlesii* of Chlorogenic was similar to gallic acid.

Figure 5A shows a graph comparing the effects obtained using the equivalent of 10 mg/mL of bagasse with chlorogenic acid and gallic acid, as well as 10 mg/mL of the bagasse extract. The graph clearly illustrates that for all three fungi analyzed, the extract exhibited a stronger inhibitory effect on fungal growth compared to the pure compounds. This demonstrates that although gallic acid plays a major biocidal role for two of the three fungi, other components in the extract actively contribute to achieving a greater inhibitory effect. In the specific case *of P. charlesii*, both compounds played a lesser role in the antifungal effect observed in the extract, suggesting that the biocidal effect found is due to other metabolites and a possible synergy among them.

To evaluate the synergistic antifungal inhibitory effect between chlorogenic acid and gallic acid, two assays were performed. In the first, a total amount of 5 mg of each compound was tested, and in the second, the amount equivalent to each standard found in 3 mg/mL of coffee pulp extract was evaluated (Fig. 5B) (0.045 mg of chlorogenic acid and 0.256 mg of gallic acid).

When we analyzed the effect of 5 mg/mL of both pure compounds we observed that this dose was lethal for *A altermata* and allowed a slight growth of *F. oxysporum*.

However, the combination of both compounds using the equivalent amount of chlorogenic acid and gallic acid found in 3 mg/mL of extract of coffee bagasse (Fig. 5B) resulted in a biocidal effect against *A. alternata* and *F. oxysporum*, the inhibitory effect conferred by the extract remains greater. For *P. charlesii*, the differences between treatments were smaller, with significance observed only between the control and the extract. These data support the hypothesis that the antifungal activity observed in the bagasse extract is only in part to these two compounds and other bioactive metabolites should be involved.

## Discussion

The total phenolic compounds content obtained in this study (91.26 mg) was higher than that reported previously [16, 17]. In contrast, other authors [12] obtained ethanolic extracts of bagasse using ultrasound-assisted extraction with values closer to those observed in this study. These results indicate that the extraction solvent plays a critical role in the recovery of total phenolic compounds, with ethanol yielding higher concentrations of these bioactive molecules.

Regarding the results obtained from the HPLC-DAD analysis, several studies have reported variable concentration of phenolic compounds and caffeine in the coffee bagasse. For example, Pérez-Hernández et al. [18] reported lower levels of ferulic acid (0.55 mg/g) and caffeine (17.39 mg/g) in an aqueous extract of dark-roasted Italian coffee from Huatusco, Veracruz, México, while the values of chlorogenic acid (11.31 mg/g) and caffeic acid (2.28 mg/g) were comparable to those found in this research. In contrast, Monente et al. [19] found a significantly higher chlorogenic acid content (36.78 mg/g) in an aqueous extract of coffee bagasse from Guatemala (Soxhlet extraction system), while the caffeine content (49.35 mg/g) was similar to that reported here. Likewise, Ramón-Gonçalves et al. [17] characterized an aqueous extract of roasted coffee from Spain (extraction by agitation) and found concentrations of 19.6 mg/g of chlorogenic acid and 20 mg/g of caffeine. The differences in phenolic

and caffeine content among studies depend on numerous factors, including the plant genetics characteristics and variety differences, as well as agronomic conditions such as climate, altitude, soil, and geographic location. The type of coffee bean processing (dry, wet, or semi-wet method), roasting level, and the material analyzed (green or roasted coffee, or a by-product such as bagasse) also play a determinant role. In addition, analytical procedures, including the type of solvent used (water, ethanol, methanol) and the extraction method (maceration, agitation, ultrasound, etc.), are determining variables in the final quantity of bioactive compounds detected. Together, these factors explain the heterogeneity observed across different studies.

Melanoidins are another group of important bioactive compounds due to their antimicrobial properties, and their presence is notable in coffee and its derivatives. However, their quantification is complex because of the lack of a reference standard that allows for accurate measurement, unlike phenolic acids. Therefore, to estimate their concentration in the ethanolic extract of coffee bagasse, an indirect methodology had to be employed. In previous studies by Tagliazucchi et al. [20] reported melanoidin contents of approximately 38 g/100 g in aqueous extracts of roasted coffee. Similarly, Pérez-Hernández et al. [18] indicated a concentration of 25 g/100 g in samples also obtained from roasted coffee. Both studies reported higher levels of melanoidins than those found in the present research. It is important to note that in both investigations, roasted coffee beans were used as the raw material for the infusions. In contrast, the present study focused on an agro-industrial by-product, coffee bagasse, which has undergone extraction processes, leading to a partial reduction in the original melanoidin content generated during the roasting of green beans. Despite this decrease, coffee bagasse still retains a significant fraction of these molecules, making it possible for it to maintain part of its biological properties. This makes it of interest for functional applications and supports its revalorization as a sustainable agro-industrial by-product.

The absence of fermentable carbohydrates detected in the extract may be directly related to the observed antimicrobial effect, as it limits the supply of essential nutrients required for microbial growth. In this regard, Casas-Godoy et al. [21] reported that the presence of high sugar levels in malt and raspberry bagasse extracts promoted yeast growth, which reduced or even masked the potential antimicrobial effect of compounds such as phenolic acids. Similarly, Ghaffar et al. [22] found that extracts from banana peels, orange peels, and potato skins showed greater microbial proliferation when they contained high concentrations of glucose, fructose, and sucrose. These results support the hypothesis that the low or absent sugar content in the ethanolic extract of coffee bagasse may contribute to the lack of microbial growth, allowing bioactive compounds, such as phenolic acids or melanoidins, to exert their antimicrobial effects in an effective way.

Several studies have shown that certain amino acids possess direct or synergistic antimicrobial properties. Tong et al. [23] reported that the amino acids cysteine, aspartic acid, and glutamic acid, in both their L and D forms, can inhibit the growth of pathogenic bacteria such as *Streptococcus mutans*, in addition to blocking the formation of bacterial biofilms, a crucial mechanism in microbial resistance. The synergistic effect observed among these amino acids suggests that their combination enhances

antimicrobial activity. In the present study, the high content of aspartic acid in the extract may be playing an active role in inhibiting microbial growth, in combination with the other amino acids present. In accordance with Elgamoudi et al. [24] found that certain amino acids, such as D-serine, D-methionine, D-tryptophan, and L-alanine, are capable of inhibiting biofilm formation by *Campylobacter jejuni*, a clinically relevant gastrointestinal pathogen. Although the exact mechanism of action is not yet fully understood, it has been proposed that these amino acids may induce morphological and structural changes in the bacterial cell wall or interfere with essential bacterial metabolic processes, ultimately suppressing proliferation. Overall, the significant presence of bioactive amino acids in the coffee bagasse extract may contribute to the observed antimicrobial effect, both through direct action against pathogenic microorganisms and by disrupting key mechanisms such as biofilm formation. This activity may include not only bactericidal effects but also potential antifungal and antiviral properties, given the structural and regulatory roles that certain amino acids can exert on different types of microorganisms.

Yosboonruang et al. [25], working with an aqueous extract from coffee plant leaves (*Coffea canephora*), reported a MIC value of 6.25 mg/mL for *S. aureus* and 12.25 mg/mL for *E. coli*. In that case, the extract was more effective at inhibiting the growth of *S. aureus*. However, the leaf extract required a lower concentration to inhibit *E. coli* growth. In our study, the highest tested concentration (20 mg/mL) barely showed signs of bacterial susceptibility, suggesting that higher concentrations could yield more favorable results. This could be because *E. coli*, being a Gram-negative bacterium, possesses an additional outer membrane composed of lipopolysaccharides and proteins, which provides greater resistance to antibacterial compounds. Gram-positive bacteria, lacking this membrane, are generally more susceptible to such compounds. Tasew et al. [13], using an aqueous extract of roasted coffee, reported a MIC of 31.25 mg/mL for both *S. aureus* and *E. coli*. In this context, our extract appears to be more effective against both bacteria. Nonetheless, the growth kinetics assay for phytopathogenic bacteria showed that both Gram-negative bacteria tested, *E. amylovora* and *D. dadantii*, were susceptible to the coffee bagasse extract. Chlorogenic acid has been identified as a compound that plays an important role in bacterial growth inhibition. This compound acts directly on the bacterial cell membrane, which is stabilized by electrostatic interactions with divalent cations, such as $Mg^{2+}$. Due to its negative surface charge, chlorogenic acid can displace these cations, destabilizing the membrane and increasing its permeability, thereby promoting the leakage of intracellular materials such as nucleic acids, proteins, and other cytoplasmic contents, ultimately leading to bacterial death [26, 27].

In a study led by Badr et al. [15], a methanolic extract of coffee bagasse was tested against *Fusarium* spp. and *Penicillium* spp., in which both genera were susceptible to the extract, showing approximately 50% of growth reduction. Their extract was rich in phenolic compounds, particularly chlorogenic and gallic acids, similar to the findings in our study, where these two compounds were also present in high concentrations. Sangta et al. [28] found that the most effective concentration of methanolic coffee pulp extract against phytopathogenic fungi was 500 mg/mL, targeting genera such as *Alternaria, Pestalotiopsis*, and *Paramyrothecium.* This value is significantly higher than the maximum concentration tested in our study (10 mg/mL), which nonetheless yielded promising results against phytopathogenic fungi, including a species of the genus *Alternaria.* Their extract was particularly rich in caffeic acid rather

than gallic or chlorogenic acids, which may explain the need for a higher concentration. As shown in our results, gallic acid played a fundamental role in fungal inhibition, especially against *A. alternata.*

## Conclusions

The ethanolic extract of coffee bagasse (*Coffea arabica*) contains a diverse profile of bioactive compounds, including phenolic acids, caffeine, melanoidins, and amino acids. The extract exhibited antibacterial activity against *Staphylococcus aureus*, *Erwinia amylovora*, and *Dickeya dadantii*, and showed concentration-dependent antifungal activity against *Alternaria alternata*, *Fusarium oxysporum*, and *Penicillium charlesii*. The antimicrobial effect of the whole extract was stronger than that of the individual phenolic compounds tested, suggesting a synergistic interaction among its metabolites. These findings highlight the potential revalorization of coffee bagasse as a sustainable agro-industrial by-product with promising applications in the control of phytopathogenic microorganisms.

## Materials and Methods

# Preparation of the Ethanolic Extract of Coffea arabica Coffee Bagasse

The sample of coffee bagasse from *Coffea arabica* was obtained from the Starbucks company in 2022 in Hermosillo, Sonora, Mexico.

The bagasse was dried in a convection oven (Heratherm, Thermo Scientific, Waltham, MA, USA) at 45°C for 12 hours. To prepare the extract, 1 g of dry sample was dissolved in 17.5 mL of an ethanol–water solution (30:70, v/v). The mixture was vortexed for 45 seconds and subjected to ultrasonic extraction (Branson 1510 sonicator, Danbury, CT, USA) for 30 minutes at 30°C. The samples were then centrifuged at 6000 × *g* for 15 minutes at 4°C (Centrifuge 16R, Thermo Scientific, MA, USA). The supernatant was vacuum filtered using Whatman No. 4 filter paper. Ethanol was removed by rotary evaporation (Labconco Digital Rotary Evaporator, Kansas City, MO, USA) at 45°C, followed by freeze-drying to remove residual water. The extract was stored at − 20°C protected from light until further analyses. All extraction steps were conducted under dark conditions to prevent photodegradation [12].

## Determination of Total Phenols and Melanoidins

Total phenol content was determined using the Folin–Ciocalteu method [12]. A standard calibration curve using serial dilutions of gallic acid (Sigma-Aldrich, USA) in 30% (v/v) ethanol was prepared. The extract concentration used was 10 mg/mL. A volume of 50 µL of sample was mixed with 3 mL of distilled water and 250 µL of 1N Folin reagent, then the mixture was left to stand for 5 minutes. Subsequently, 750 µL of 7.5% $Na_2CO_3$ and 950 µL of distilled water were added. The reaction mixture was incubated for 30 minutes at room temperature. Absorbance was measured at 765 nm.

A solution of 10 mg of ethanolic coffee bagasse extracted in 10 mL of distilled water was prepared to obtain a final concentration of 1 mg/mL. A 1:10 dilution of this solution was then made, and absorbance was measured at 420 nm (Spectrophotometer Cary 100, Varian Australia Pty Ltd, Belrose, NSW, Australia). Total melanoidin content was calculated using the specific extinction coefficient reported by Tagliazucchi et al. [20]. All samples were analyzed by triplicate.

# Identification and Quantification of Phenolic Compounds and Caffeine by Reverse-Phase HPLC-DAD

The identification and quantification of phenolic compounds and caffeine in the *Coffea arabica* coffee bagasse extract by HPLC-DAD was carried out following the method described by Cantos et al. [29]. An HPLC system with diode array detection (HPLC-DAD, model 1260 Infinity, Agilent Technologies) was used. Separation of bioactive compounds was performed using a C-18 reverse-phase column (5 µm, 25 cm × 4 mm, Supelcosil™ LC-18, SUPELCO) at a flow rate of 1 mL/min. The mobile phase consisted of solvent A (water with 5% formic acid) and solvent B (HPLC-grade methanol). A sample volume of 150 µL was injected, with a run time of 55 minutes.

Compound identification was based on retention times and spectra from 13 commercial standards: caffeine, chlorogenic acid, caffeic acid, gallic acid, sinapic acid, *p*-coumaric acid, ferulic acid, vinyl acid, catechin, epicatechin, 4-hydroxybenzoic acid, 2,5-dihydroxybenzoic acid, and syringic acid (Sigma-Aldrich, USA).

# Determination of Sugars and Amino Acids

High-performance anion-exchange liquid chromatography with electrochemical detection (DIONEX ICS3000) was used. The equipment was fitted with a Dionex CarboPac PA20 column (3 × 150 mm) and a Dionex CarboPac PA20 Guard precolumn (3 × 30 mm) with a flow rate of 0.4 mL/min. The mobile phases used were Milli-Q water and sodium hydroxide (isocratic elution at 16 mM NaOH, ultrapure grade). The system was equipped with a DIONEX AS40 autosampler [30]. The quantified sugars were glucose (Duchefa G0802), fructose (Panreac A3688), sucrose (Duchefa S0809), maltose (Duchefa M0811), galactose (Sigma G5388), mannitol (Duchefa M0803), trehalose (Sigma T0167), raffinose (Sigma R0250), and mannose (Sigma M4625). A calibration curve was created using concentrations of 10 µM, 20 µM, 50 µM, and 100 µM of each sugar studied. Three chromatograms were obtained for each calibration point and sample. Sugar analysis was performed on the ethanolic extract of coffee bagasse.

The method used for amino acids determination allows the separation and quantification of 18 amino acids (Asp, Glu, Ser, Asn, Gly, Gln, His, Thr, Ala, Arg, GABA, Pro, Tyr, Val, Ile, Leu, Lys, and Phe). Calibration was performed using Sigma-Aldrich amino acid standards (AAS18). Both standards and samples were derivatized with the Waters AccQ-Tag Ultra Derivatization Kit (ref. 186003836), which includes a borate buffer and derivatizing agent, all mixed with the sample in high-recovery HPLC vials. Samples were analyzed by reverse-phase HPLC with fluorescence detection using a high-performance liquid chromatograph (Alliance 2695, Waters) equipped with an AccQ-Tag column (3.9 × 150 mm), with a flow

rate of 1 mL/min, and a fluorescence detector (Waters 2475 Multi-λ Fluorescence Detector). Elution was carried out with a gradient of sodium acetate (140 mM, pH 5.8), HPLC-grade acetonitrile, and water. The excitation wavelength was 250 nm, and the emission wavelength was 395 nm [30]. This analysis was conducted on the ethanolic extract of coffee bagasse. All the analyses were performed in triplicate.

# Determination of Antibacterial Activity

The broth microdilution technique was used for Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) testing, employing a 96-well round-bottom microplate to evaluate different concentrations of ethanolic extract of coffee bagasse. The extract was dissolved in Mueller-Hinton broth at concentrations of 2, 5, 8, 11, 14, 17, and 20 mg/mL. In each well, 5 µL of inoculum, adjusted to a concentration of $1 \times 10^8$ CFU/mL, was added. The positive control consisted of Mueller-Hinton broth with bacterial inoculum, while the negative control contained only broth. The dilutions were incubated for 24 hours at 37°C. After this period, 20 µL from each treatment was transferred (in duplicate) to Mueller-Hinton agar Petri dishes and incubated for an additional 24 hours at 37°C. The MIC was defined as the lowest extract concentration that caused visible inhibition of bacterial growth, while the MBC was the lowest concentration that inhibited 99.9% of bacterial growth. This assay was conducted on *Staphylococcus aureus* (ATCC 6538P) and *Escherichia coli* (ATCC 25922), responsible for foodborne illness (Table S2). Additionally, the MIC and MBC of standard phenolic compounds, gallic acid and chlorogenic acid, were tested at concentrations of 0.5, 1, 2, 3, 4 and 5 mg/mL, following the same procedure used for the extract.

To evaluate the effect of the ethanolic extract of coffee bagasse on phytopathogenic bacteria over a defined period, the strains *Erwinia amylovora* (CECT 222) and *Dickeya dadantii* (CFBP 3855) were used (Table S2). The microplate technique was employed to monitor bacterial behavior over 48 hours while exposed to the following extract concentrations: 5, 10, 15, and 20 mg/mL, prepared from a 100 mg/mL stock solution. The extract was dissolved in LB medium. Bacterial density was adjusted to an optical density of 0.05. The experiment was performed in triplicate. As a negative control, Luria Bertani (LB) medium without any extract was used. Chlorogenic acid and gallic acid standard compounds, identified as the most abundant phenolic compounds in the extract, were selected to evaluate their individual biocidal effects and their mix at equivalent amounts corresponding to the content in 15 and 20 mg/mL of extract were tested on the phytopathogenic bacteria tested.

# Determination of Antifungal Activity

To assess the antifungal activity of the ethanolic extract of coffee bagasse, six phytopathogenic fungal strains were used: *Alternaria alternata* (CECT 20192), *Penicillium aurantiogriseum* (CECT 20226), *Botrytis cinerea* (CECT 20754), *Penicillium digitatum* (CECT 20796), *Fusarium oxysporum* (CECT 20420), and *Penicillium charlesii* (CECT 2275), from the fungal collection of the Institute of Agrobiotechnology (Navarra, Spain) (Table S2).

In the first antifungal test, the biocidal effect of the extract was evaluated against the six fungi at initial concentrations of 0.2, 0.4, and 1.0 mg/mL. The extract was directly diluted into the culture medium and

then poured into each plate. The plates were perforated in the center with a 5 mm diameter punch, and a fungal plug was placed in the hole. The experiment was conducted in triplicate for all six fungi. As a control, potato dextrose agar (PDA) plates without any extract were used. In this preliminary assay, an inhibitory effect on the growth of *A. alternata, F. oxysporum*, and *P. charlesii* was observed, which led to the selection of these fungi for subsequent experiments.

In the follow-up antifungal assays, the same methodology was applied, but the extract concentrations were increased to 2, 3, 5, and 10 mg/mL. The extract was incorporated into the PDA medium before solidification. Fungal growth was evaluated after 72 hours of incubation at 27°C in complete darkness. Colony diameters were measured in each treatment using a ruler to assess the extent of fungal inhibition.

Chlorogenic acid and gallic acid standard compounds, identified as the most abundant phenolic compounds in the extract, were selected to evaluate their individual biocidal effects. Based on HPLC quantification, equivalent amounts corresponding to the content in 10 and 100 mg of extract were tested. These concentrations were 0.153 and 1.53 mg/mL for chlorogenic acid and 0.85 and 8.55 mg/mL for gallic acid, respectively.

To evaluate a possible synergistic effect between the two phenolic standards, two additional tests were performed. In the first, 5 mg of chlorogenic acid standard and 5 mg of gallic acid standard were added to the culture medium, corresponding to the amounts present in approximately 326 mg and 58.42 mg of extract, respectively. In the second test, the equivalent amounts of these acids found in 3 mg of extract were tested (0.045 mg/mL chlorogenic acid and 0.256 mg/mL gallic acid). The plates were incubated at 27°C in complete darkness for 72 hours. Fungal growth was assessed by measuring colony diameter using a ruler. All tests were carried out in triplicate.

# Statistical Analysis

All data were subjected to normality and homoscedasticity tests. Results are expressed as the mean ± standard deviation from three independent replicates. Significant differences among means were assessed using analysis of variance (ANOVA) followed by a post hoc test (Duncan or Tukey), with a significance of $p < 0.05$. A factorial design was used for the antifungal activity tests.

# Declarations

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**Author contribution**

K.C and N.G.M conceived and designed the research. R.C.D, A.B.N and K. C. conducted the experiments. R.C.D, K. C., E.B, and N.G.M. analyzed the data. R.C.D and K. C. wrote the manuscript. E.B, C.B, K.C and N. G.M. participated in drafting and critically revising the article. All authors read and approved the manuscript.

**Data availability**

The original contributions presented in this study are included in the article; further inquiries can be directed to the corresponding author.

**Ethical approval**

This article does not contain any studies with human participants or animals performed by any of the authors.

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**Conflict of interests**

The authors declare no conflict of interests.

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# Tables

**Table 1.** Caffeine and phenolic compounds concentration (mg/g dry extract)in the ethanolic extract of *Coffea arabica* coffee bagasse obtained by HPLC-DAD at the indicates absorbances.

| **Compound** | **Absorbance ($\lambda_{máx}$)** | **Ethanolic extract (mg/g dry extract)** |
|---|---|---|
| Caffeine | 270 | 47.65 ± 0.53 |
| Chlorogenic acid | 320 | 15.31 ± 0.09 |
| Caffeic acid | 320 | 2.73±0.04 |
| Gallic acid | 270 | 85.58 ± 11.20 |
| Sinapic acid | 320 | 2.08 ± 0.02 |
| *p*-Coumaric acid | 320 | 1.67 ± 0.00 |
| Ferulic acid | 320 | 1.22 ± 0.01 |
| Vinyl acid | 260 | ND |
| Catechin | 280 | ND |
| Epicatechin | 280 | ND |
| 4-Hydroxybenzoic acid | 260 | ND |
| 2,5-Dihydroxybenzoic acid | 330 | ND |
| Syringic acid | 270 | 1.26 ± 0.01 |

Results are expressed as the mean of three replicates ± standard deviation.

ND: not detected

**Table 2.** Amino acid concentration (pmol/mg coffee bagasse) in the ethanolic extract of *Coffea arabica* coffee bagasse obtained by HPLC with fluorescence detection. Results are expressed as the mean of three replicates ± standard deviation.

| Amino acid | pmol/mg of bagasse extract |
|---|---|
| Asp | 9.68 ± 0.51 |
| Glu | 1.91 ± 0.25 |
| Ser | 34.11 ± 2.33 |
| Asn | 2.87 ± 0.57 |
| Gly | 7.82 ± 0.44 |
| Gln | ND |
| His | 2.49 ± 0.68 |
| Thr | 1.26 ± 0.21 |
| Ala | 9.72 ± 0.81 |
| Arg | ND |
| GABA | 14.51 ± 3.17 |
| Pro | 4.59 ± 1.41 |
| Tyr | 1.64 ± 0.10 |
| Val | 1.91 ± 0.12 |
| Met | ND |
| Ile | 2.03 ± 0.15 |
| Leu | 1.76 ± 0.17 |
| Lys | 2.99 ± 0.28 |
| Phe | 1.70 ± 0.08 |

# Figures

**Figure 1.**

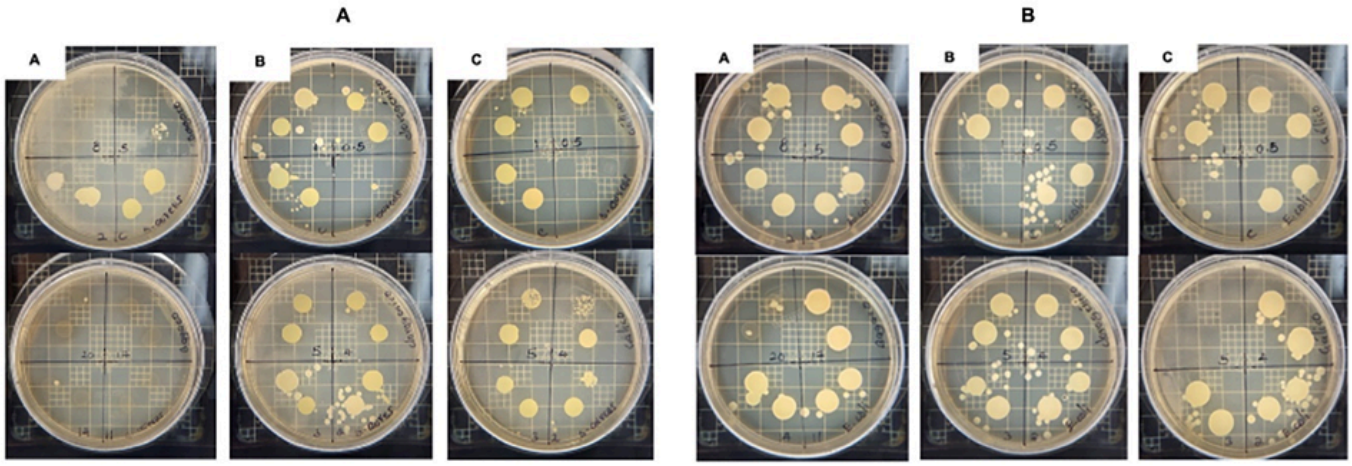



**Figure 1**

**A:** Results of MIC and MBC methods for *S. aureus*. A: Ethanolic coffee bagasse extract (2, 5, 8, 11, 14, 17, 20 mg/mL); B: Chlorogenic acid (0.5, 1, 2, 3, 4, 5 mg/mL); C: Gallic acid (0.5, 1, 2, 3, 4, 5 mg/mL). **B:** Results of MIC and MBC methods for *E. coli*. A: Ethanolic extract (2, 5, 8, 11, 14, 17, and 20 mg/mL); B: Chlorogenic acid (0.5, 1, 2, 3, 4, 5 mg/mL); C: Gallic acid (0.5, 1, 2, 3, 4, 5 mg/mL).

# Figure 2.

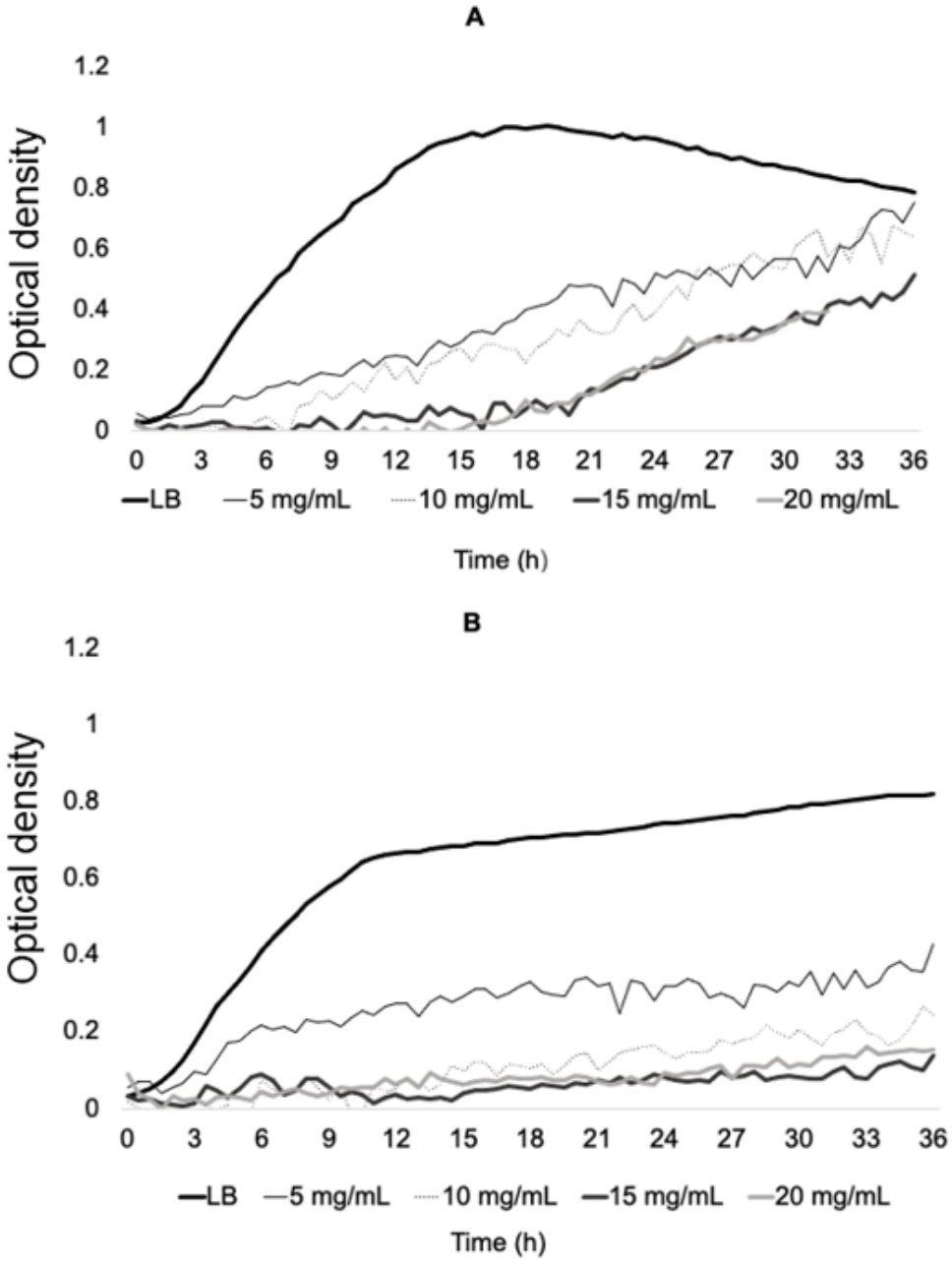



**Figure 2**

Growth kinetics of phytopathogenic bacteria's *E. amylovora* (**A**) and *D. dadantii* (**B**) over 24 hours. Control: LB medium. Concentrations: 5, 10, 15, and 20 mg/mL of coffee bagasse extract.

# Figure 3.

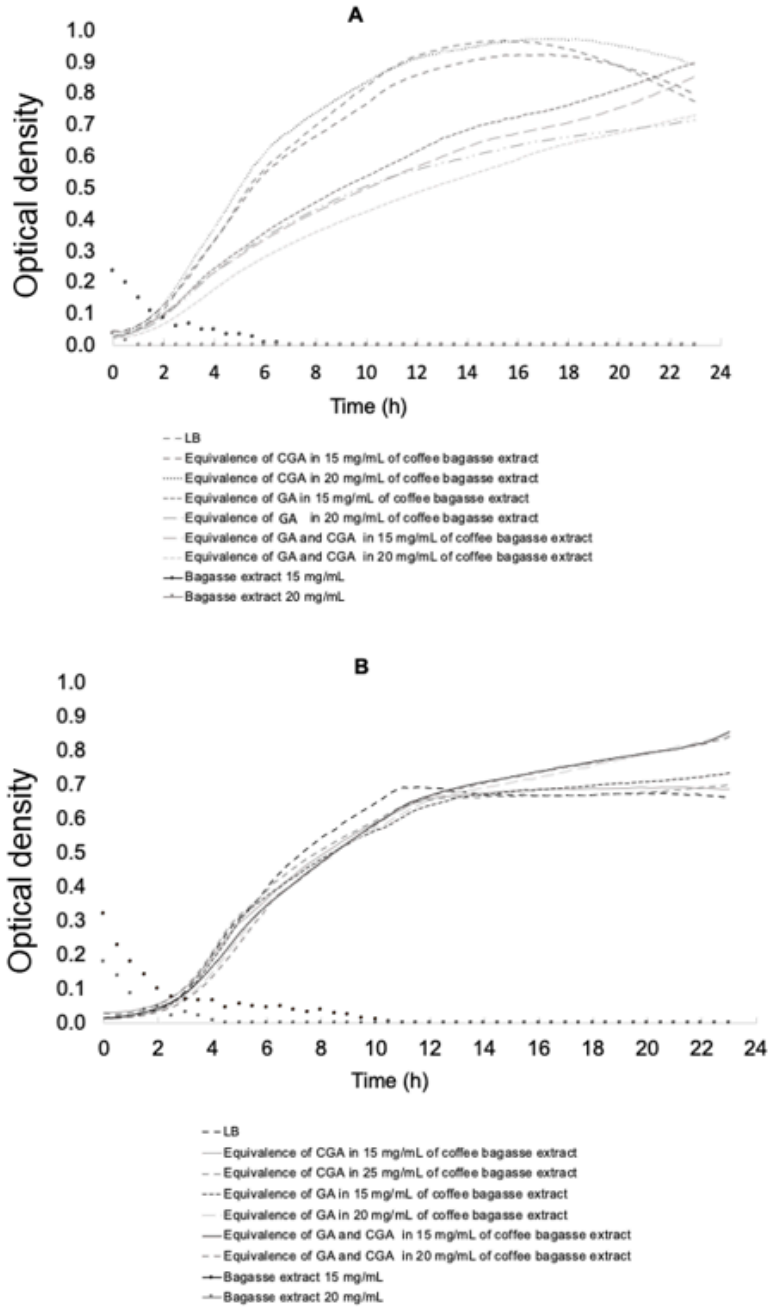
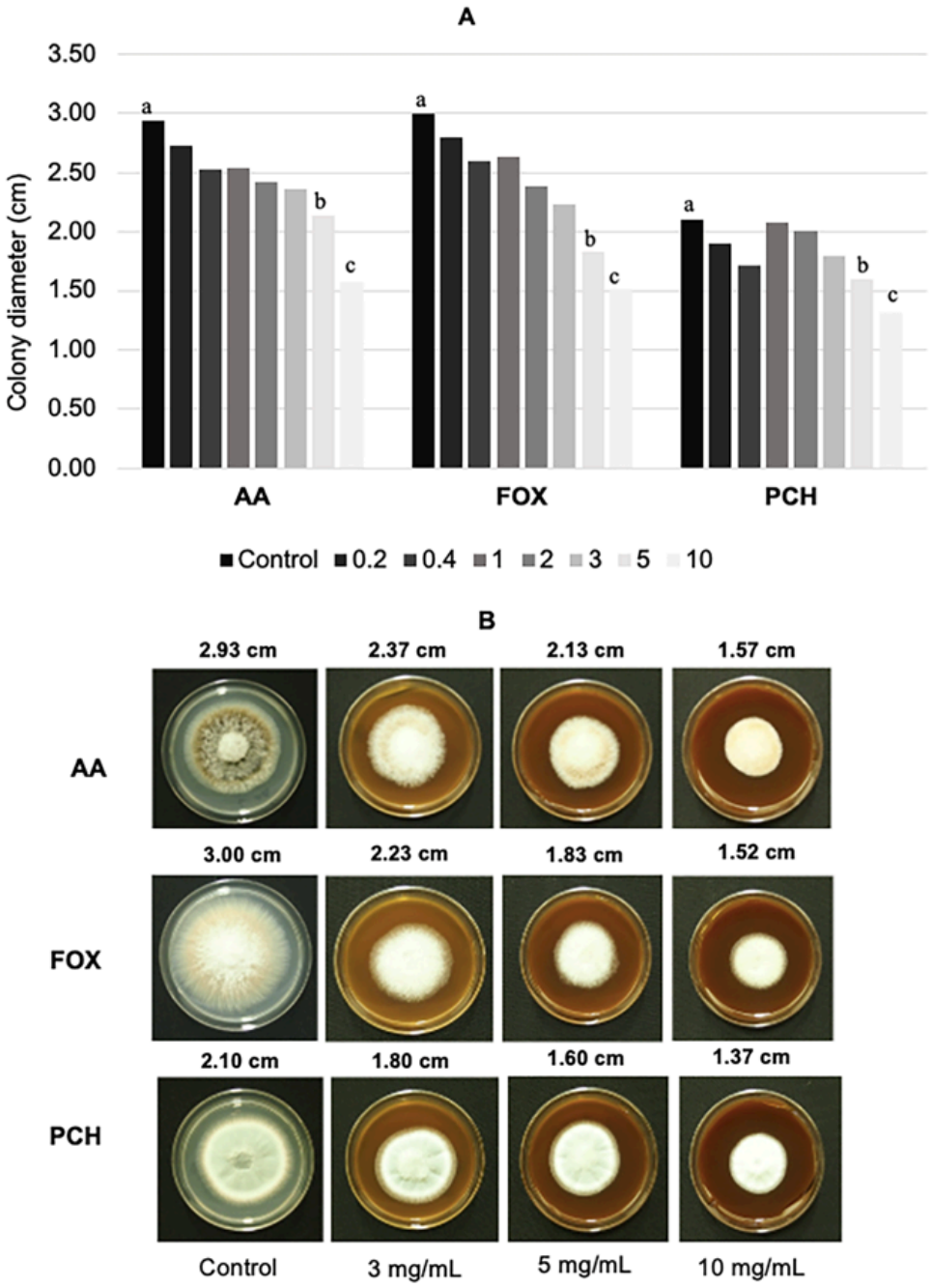



**Figure 3**

Growth kinetics of the phytopathogenic bacteria *E. amylovora* (**A**) and *D. dadantii* (**B**) over 24 h under different treatments. The evaluated conditions included: LB medium as control; gallic acid (GA) and chlorogenic acid (CGA), individually and in combination (GA + CGA), at concentrations equivalent to their respective contents in 15 and 20 mg/mL of ethanolic coffee bagasse extract; and the ethanolic coffee bagasse extract at 15 and 20 mg/mL.

**Figure 4.**




## Figure 4

Antifungal effect of the coffee bagasse extract at **A)** Bar graph shows the different fungi growth at concentrations of 0.2, 0.4, 1, 2, 3, 5 and 10 mg/mL. Results represent the mean of three replicates. Bars with different letters denote significant differences (Tukey $P < 0.05$). **B)** Fungi growth at the highest concentrations tested: 3, 5 and 10 mg/mL. **AA**: *A. alternata*; **FOX**: *F. oxysporum*; **PCH**: *P. charlesii*.

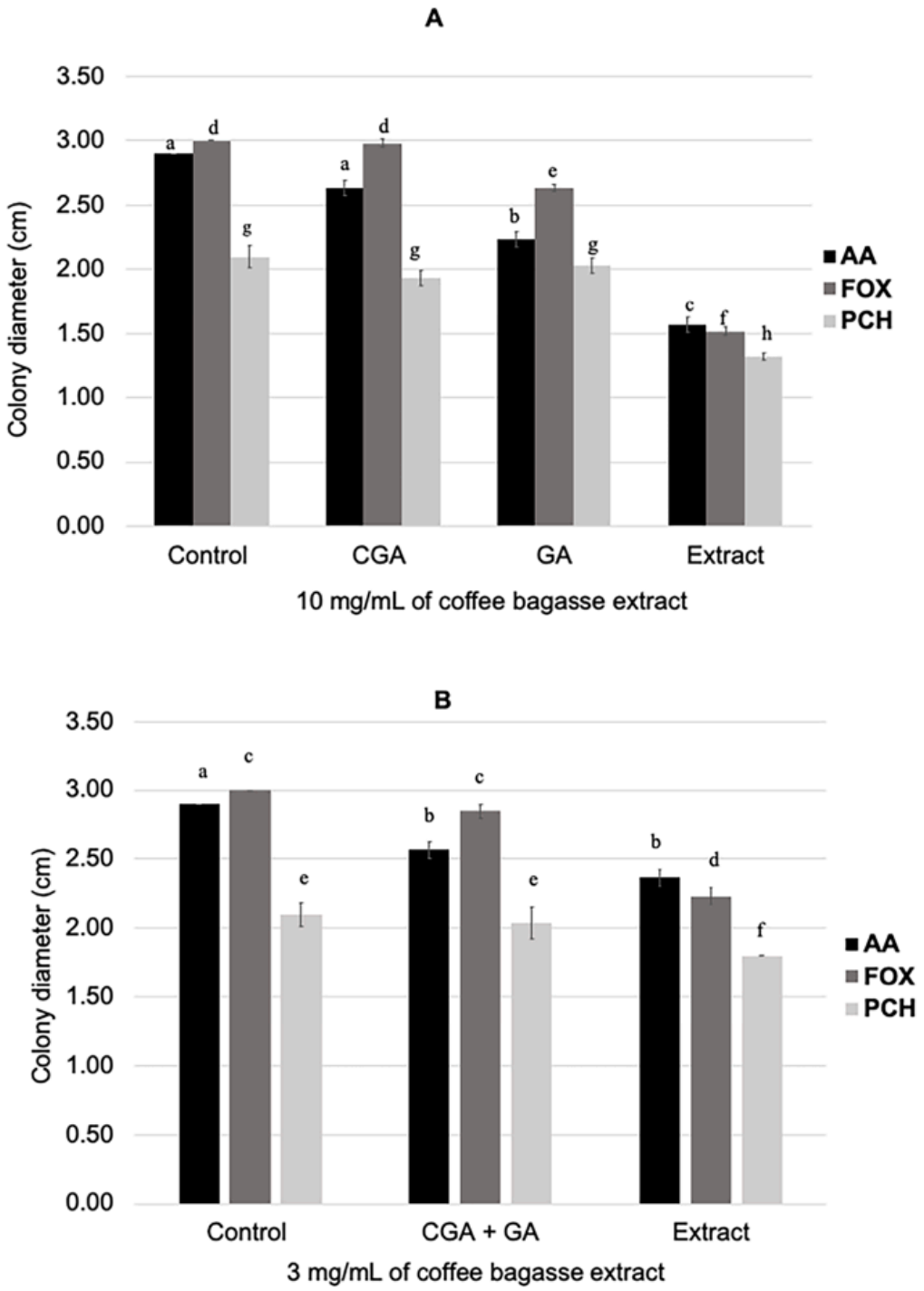



**Figure 5**

**A:** Antifungal activity of 10 mg/mL of pure extract and the corresponding content of chlorogenic acid (CGA) and gallic acid (GA) standard compounds in 10 mg/mL of extract. **B:** Antifungal activity of 3 mg/mL of extract and the equivalent amount of chlorogenic acid and gallic acid in 3 mg/mL of extract. **AA**: *A. alternata*; **FOX**: *F. oxysporum*; **PCH**: *P. charlesii*. The result represents the mean of three replicates. Different letters denote significant differences ($p < 0.05$ between treatments.

## Supplementary Files

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