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Bioactive Potential, Gastrointestinal Bioaccessibility, and Preliminary Safety Assessment of a Phenolic-Rich Coffee Pulp (*Coffea arabica*) Extract

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Abstract

Background/Objectives: Coffee pulp (*Coffea arabica*) is an agroindustrial byproduct of coffee production and has high potential as a source of bioactive compounds. **Methods:** In this study, a coffee pulp extract was characterized by determining its total polyphenol content (TPC), condensed tannins (CT), and total flavonoids (TFC), as well as its antioxidant activity, inhibition of α-amylase and α-glucosidase, stability during in vitro gastrointestinal digestion, and toxicity in *Artemia salina*. **Results:** The extract exhibited a TPC of 90.33 mg GAE/g of extract, a TFC of 60.48 mg CE/g of extract, and a CT of 27.27 mg PC1eq/g of extract. Antioxidant activity was measured as follows: DPPH (105.41 mg TE/g of extract), ABTS (69.41 mg TE/g of extract), TAA (325.11 mg TE/g of extract), and FRAP (106.03 mg TE/g of extract). The extract inhibited α-amylase by 20.67% and α-glucosidase by 34.69% at 1000 µg/mL. In vitro digestion reduced phenolic compounds and antioxidant activity, with a low proportion of condensed tannins considered absorbable, while polyphenols could not be detected in the dialysis. On the other hand, the median lethal concentration (LC_{50}) in *A. salina* was > 1000 µg/mL, thus considered non-toxic. **Conclusions:** These results demonstrate the potential of coffee pulp as a source of safe bioactive compounds, although its stability and gastrointestinal availability must be considered for its utilization.

Keywords: digestion; procyanidins; waste

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1. Introduction

Coffee is one of the most important crops; in fact, it is the second most traded commodity in the world, second only to crude oil [1]. Global production has been around 10 million tons per year [2]. The most widely cultivated species are *Coffea arabica* and *Coffea canephora* (or *Coffea robusta*), which account for approximately 60% and 40% of global production, respectively [3]. Coffee processing generates a large amount of waste consisting of husks, pulp, mucilage, and coffee grounds. Coffee pulp represents 40–50% of this waste, resulting

in millions of metric tons of waste generated annually [4]. However, proper management of this waste is complicated by the high volumes and a lack of specialized infrastructure, leading to serious environmental problems, as tannin-rich leachates can contaminate soil and water, and the high organic load promotes the proliferation of microorganisms [5–7].

In terms of the circular bioeconomy, coffee pulp is an interesting material, as it is a rich source of phenolic compounds, such as chlorogenic acid, ferulic acid, flavonoids, and condensed tannins, such as procyanidins. These compounds have been extensively studied because they are associated with health benefits when consumed in foods rich in these components. Evidence shows that molecules such as caffeic acid, chlorogenic acid, polyphenols, and procyanidins possess antioxidant, anti-inflammatory, antimicrobial, and antidiabetic properties, as well as neuroprotective effects [4]. Consequently, the valorization of these types of byproducts has become a research priority, offering an alternative to their improper disposal and potentially generating economic and health benefits in coffee-growing regions.

However, when consumed, these bioactive compounds undergo significant modifications during gastrointestinal digestion due to changes in pH, enzymatic activity, and interactions with the digestive environment. Therefore, the bioactivity observed in in vitro assays does not necessarily reflect the amount or activity of the compounds that remain available after digestion [8]. Furthermore, information on the safety of coffee pulp extracts remains limited. In this regard, the objective of this study was to characterize the phytochemical profile and antioxidant capacity of a coffee pulp extract rich in phenolic compounds, evaluate its inhibitory activity against α-amylase and α-glucosidase, determine the stability and bioaccessibility of the compounds through in vitro digestion, and assess their preliminary safety.

2. Materials and Methods

2.1. Plant Material

The coffee pulp (*Coffea arabica*) was obtained from coffee producers in Xilitla, San Luis Potosí, Mexico. The pulp was dried by convection at 60 °C (Quincy Lab, Burr Ridge, IL, USA) for 48 h. The pulp was ground using an electric grinder (Nima, model 8300, 200W, Jinhua, China) and sieved through a #40 mesh sieve until a powder with a particle size of <420 µm was obtained. The sample was stored at 25 °C, protected from light.

2.2. Reagents

Gallic acid, trolox, catechin, procyanidin C1, ABTS (2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)), DPPH (2,2-diphenyl-1-picrylhydrazyl), Folin–Ciocalteu reagent, Na_2CO_3, $NaNO_2$, heat-stable α-amylase from *Bacillus* sp., α-glucosidase from *Saccharomyces cerevisiae*, DNS (3,5-dinitrosalicylic acid), TPTZ (2,4,6-tris(2′-pyridyl)-1,3,5-triazine), and potassium persulfate were purchased from Merck Sigma-Aldrich (St. Louis, MO, USA). Acetone, ethanol, butanol, HCl, NaOH, sulfuric acid, and NaCl were obtained from Jalmek Científica (San Nicolás de los Garza, Nuevo León, Mexico). pNPG (4-nitrophenyl β-D-glucopyranoside) was purchased from ChemCenter (La Jolla, CA, USA). All other chemicals and solvents were of analytical grade.

2.3. Obtaining Coffee Pulp Extract

One g of coffee pulp powder was mixed with 93 mL of 60% acetone in glass recipients. Extraction was performed using an ultrasonic bath (Branson 2510, Danbury, CT, USA) at 40 kHz and 40 °C for 15 min. The samples were then filtered through 11-µm filter paper, and the extracts were concentrated under reduced pressure using a rotary evaporator (IKA RV10, Staufen, Germany) to remove the acetone. The concentrated aqueous extract was

then dried in an oven (Quincy Lab, Burr Ridge, IL, USA) at 40 °C for 72 h to remove residual water and obtain the dry extract. Finally, the dry extracts were stored in light-protected, sealed containers and refrigerated at 5 °C for subsequent analysis.

2.4. Phytochemical Characterization

2.4.1. Total Polyphenol Content

The total polyphenol content was determined using the Folin–Ciocalteu method [9]. 20 µL of the sample (1000 µg/mL, prepared in ethanol 50%) were mixed with 20 µL of the Folin–Ciocalteu reagent and allowed to react for 5 min. Later, 20 µL of Na_2CO_3 (0.01 M) were added and mixed and left to react for 5 min. To finish, 125 µL of distilled water were added, and the absorbance was measured at 790 nm using a microplate reader (BIOBASE-EL10A ELISA Reader, Jinan, China). The results were expressed as milligrams of gallic acid equivalents per gram of extract (mg GAE/g).

2.4.2. Condensed Tannin Content

The condensed tannin content was determined using the HCl-butanol method with modifications [10]. Briefly, 125 µL of the sample (1000 µg/mL, prepared in ethanol 50%) was placed in glass tubes. Next, 750 µL of HCl-butanol (1:9) and 25 µL of ferric reagent (2% $NH_4Fe(SO_4)_2$ in 2 N HCl) were added. The tubes were sealed and placed in a boiling water bath (95 °C) for 1 h. After cooling, 200 µL was transferred to a microplate, and the absorbance was measured at 550 nm using a microplate reader (BIOBASE-EL10A ELISA Reader, Jinan, China). The results were expressed as milligrams of procyanidin C1 equivalents per gram of extract (mg PC1eq/g).

2.4.3. Flavonoid Content

The flavonoid content was determined according to the method reported by Aranda-Ledesma et al. [11]. First, 31 µL of sample (1000 µg/mL, prepared in ethanol 50%) was added and mixed with 9.3 µL of 5% (*w*/*v*) $NaNO_2$ and 9.3 µL of distilled water. The mixture was shaken and incubated for 5 min. Then, 9.3 µL of 10% (*w*/*v*) $AlCl_3$ was added to the mixture and incubated for 3 min. Finally, 125 µL of 0.5 M NaOH was added, and the mixture was incubated for 30 min in the dark at 25 °C. Absorbance was measured at 510 nm using a microplate reader (BIOBASE-EL10A ELISA Reader, Jinan, China). The results were expressed as milligrams of catechin equivalents per gram of extract (mg CE/g).

2.5. Antioxidant Activity

2.5.1. DPPH Radical Scavenging

The DPPH assay was performed according to Ramírez-Esparza et al. [9]. The DPPH radical was prepared at 60 µM in ethanol. Subsequently, 193 µL of the DPPH radical and 7 µL of the sample (1000 µg/mL, prepared in ethanol 50%, and diluted 1:10) were mixed in a microplate well. The mixture was allowed to react for 30 min at 25 °C, protected from light. Absorbance was measured at 517 nm using a microplate reader (BIOBASE-EL10A ELISA Reader, Jinan, China). Results were expressed as milligrams of Trolox equivalents per gram of extract (mg TE/g).

For in vitro digestion, the results were reported as a percentage of inhibition according to the following Equation (1):

$$DPPH\ Inhibition\ (\%) = \frac{\left(Absorbance_{Control} - Absorbance_{Sample}\right)}{Absorbance_{Control}} \times 100. \quad (1)$$

2.5.2. ABTS Radical Scavenging

The ABTS assay was performed according to Espitia-Hernández et al. [12]. The ABTS radical was prepared using a solution of equal parts ABTS (7 mM) and $K_2S_2O_8$ (2.45 mM) in water. The mixture was allowed to stand in the dark at 25 °C for 14 h before use. Subsequently, a portion of the solution was taken and mixed with ethanol, and the absorbance was adjusted to 0.7 ± 0.02 nm. Next, 190 µL of the ABTS radical was mixed with 10 µL of the sample (1000 µg/mL, prepared in ethanol 50%) in a microplate well and read immediately. Absorbance was measured at 734 nm using a microplate reader (BIOBASE-EL10A ELISA Reader, Jinan, China). The results were expressed as mg TE/g of extract.

2.5.3. Total Antioxidant Activity (TAA) Assay

The TAA method was developed using the ammonium molybdate assay [13]. In summary, 100 µL of sample (1000 µg/mL, prepared in ethanol 50%) was mixed with 1 mL of phosphomolybdenum solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The test tubes were covered with aluminum foil and incubated in a water bath at 95 °C for 90 min. Then, the tubs were allowed to cool at room temperature. Absorbance was measured at 765 nm using a microplate reader (BIOBASE-EL10A ELISA Reader, Jinan, China). The results were expressed in mg TE/g of extract.

2.5.4. Ferric Reducing Antioxidant Power (FRAP)

The FRAP assay was performed as described by Torres-León et al. [14]. The FRAP reagent was prepared by mixing acetate buffer (0.3 M at pH 3.6), $FeCl_3$ (20 mM), and 2,4,6-tris(2-pyridyl)-s-triazine (TPTZ) (10 mM in 40 mM HCl). 10 µL of sample (1000 µg/mL, prepared in ethanol 50%) was mixed with 290 µL of FRAP reagent in a microplate. The mixture was incubated at 37 °C for 15 min. Absorbance was measured at 593 nm using a microplate reader (BIOBASE-EL10A ELISA Reader, Jinan, China). The results were expressed as mg TE/g of extract.

2.6. Inhibition of Carbohydrate-Hydrolyzing Enzymes

2.6.1. Inhibition of α-Amylase

The α-amylase inhibition assay was performed according to the methodology described by Oluwagunwa et al. [15] with slight modifications. α-amylase was prepared at 25 U/mL in 20 mM phosphate buffer (pH 7). The extract was dissolved in phosphate buffer at 1000 µg/mL. Potato starch (1.0% *w*/*v*) was dissolved in phosphate buffer and placed in a boiling water bath until a clear solution was obtained. Next, 50 µL of the sample (1000 µg/mL, prepared in phosphate buffer) and 50 µL of α-amylase (25 U/mL) were added to the test tubes and incubated at 37 °C for 10 min. After incubation, 50 µL of 1.0% potato starch was added, and the mixture was incubated at 37 °C for 10 min. The reaction was completed by adding 100 µL of DNS (DNS: 0.8 g NaOH/15 g $KNaC_4H_4O_6 \cdot 4H_2O$/0.5 g DNS in 50 mL water), followed by incubation in a boiling water bath at 100 °C for 5 min. The reaction mixture was allowed to cool, and 1500 µL of water was added. Finally, 200 µL of the mixture was transferred to a microplate well, and the absorbance was measured at 540 nm using a microplate reader (BIOBASE-EL10A ELISA Reader, Jinan, China). Acarbose (1000 µg/mL) was used as a positive control; a negative control (uninhibited reaction) and a blank (without enzyme) were also run. The percentage of inhibition was calculated using the following Equation (2):

$$\textit{Inhibition of α-amylase } (\%) = \frac{(A_C - A_{cb}) - (A_s - A_{sb})}{(A_c - A_{cb})} \times 100, \quad (2)$$

where A_c is the absorbance of the negative control, A_{cb} is the absorbance of the control without the enzyme (blank), A_s is the absorbance of the sample (inhibited reaction), and A_{sb} is the absorbance of the sample without the enzyme (blank).

2.6.2. Inhibition of α-Glucosidase

The α-glucosidase inhibition assay was performed according to the methodology described by Kazeem et al. [16] with slight modifications. The α-glucosidase was prepared at 10 U/mL in 20 mM phosphate buffer (pH 7.0). The substrate p-nitrophenyl-α-D-glucopyranoside (pNPG) was prepared at 1.5 mM in the same buffer. 50 µL of the sample (1000 µg/mL, prepared in phosphate buffer) was mixed with 100 µL of α-glucosidase (1.0 U/mL), and the mixture was preincubated at 37 °C for 10 min. Subsequently, 50 µL of pNPG (1.5 mM) was added, and the mixture was incubated again at 37 °C for 20 min. The reaction was stopped by adding 200 µL of 0.1 M Na_2CO_3. Finally, 200 µL of the reaction was transferred to a microplate well. Absorbance was measured at 405 nm using a microplate reader (BIOBASE-EL10A ELISA Reader, Jinan, China). Acarbose (1000 µg/mL) was used as a positive control; a negative control (uninhibited reaction) and a blank (without enzyme) were also run. The percentage of inhibition was calculated according to the following Equation (3):

$$\textit{Inhibition of α-glucosidase } (\%) = \frac{(A_C - A_{cb}) - (A_s - A_{sb})}{(A_c - A_{cb})} \times 100, \quad (3)$$

where A_c is the absorbance of the negative control, A_{cb} is the absorbance of the control without the enzyme (blank), A_s is the absorbance of the sample (inhibited reaction), and A_{sb} is the absorbance of the sample without the enzyme (blank).

2.7. In Vitro Gastrointestinal Digestion

The in vitro digestion was performed continuously and sequentially, following the methodology reported by Julián-Flores et al. [17] with modifications. 100 mg of the dry extract was dissolved in 10 mL of distilled water. The initial pH of the extract was adjusted to 5.6–6.9 using 0.1 M NaOH. The oral phase was simulated by adding 0.3 mL of α-amylase (100 U/mL in 1 mM $CaCl_2$) to the sample, followed by incubation at 37 °C for 2 min under orbital agitation (Innova 44, Eppendorf/New Brunswick Scientific, Hamburg, Germany). Subsequently, gastric digestion was performed by readjusting the pH to 2.0 with 1 M HCl and adding pepsin (25 mg/mL in 0.1 N HCl) at a rate of 0.05 mL per 1 mL of sample, followed by incubation for 1 h at 37 °C and 130 rpm. Next, for intestinal digestion, the pH was adjusted to 6.0 with 1 M $NaHCO_3$ and a commercial enzyme preparation (1.33 g in 100 mL $NaHCO_3$, Zymeton®, Laboratorios Química Son's, S.A. de C.V., Puebla, Mexico) was added as a source of pancreatin and bile salts at a rate of 0.25 mL per 1 mL of sample, followed by incubation for 2 h at 37 °C and 45 rpm. Finally, intestinal absorption was simulated using Spectra/Por dialysis membranes (MWCO 6–8 kDa; Spectrum Laboratories, Rancho Dominguez, CA, USA), by placing 2 mL of the intestinal digest inside the membrane and immersing it in 200 mL of 50 mM phosphate buffer (pH 7–7.5) at 37 °C with orbital shaking for 24 h at 150 rpm. The dialyzable fraction ("OUT") was considered potentially absorbable, while the fraction remaining inside the membrane ("IN") represented the non-absorbable compounds. Aliquots (1 mL) were collected at the end of each digestive phase and stored in the dark at −20 °C until further analysis. Total polyphenols, condensed tannins, and antioxidant activity (DPPH) were monitored as representative indicators of the stability and potential bioaccessibility of the phenolic compounds, following the methodologies described in Sections 2.4.1, 2.4.2 and 2.5.1, using aliquots from each phase.

2.7.1. Bioaccessibility Index

The effect of in vitro gastrointestinal digestion on total phenolic content and condensed tannins was evaluated using the gastrointestinal bioaccessibility index (BI_{GI}) [18]. Bioaccessibility was calculated as the percentage of the initial content recovered after the intestinal digestion phase with the following Equation (4):

$$BI_{GI}(\%) = \frac{C_{Intestinal}}{C_{Initial}} \times 100, \quad (4)$$

where $C_{intestinal}$ is the content of total polyphenols or condensed tannins measured after the intestinal digestion phase and $C_{initial}$ is the corresponding content in the original sample.

2.7.2. Dialyzable Fraction

The fraction potentially available for absorption was evaluated through dialysis [19]. The dialyzable fraction (DF) was calculated as the percentage of the compounds present in the intestinal fraction that passed through the dialysis membrane with the following Equation (5):

$$DF\ (\%) = \frac{C_{OUT}}{C_{Intestinal\ IN}} \times 100, \quad (5)$$

where C_{OUT} represents the content of total polyphenols or condensed tannins detected outside the dialysis membrane and $C_{intestinal\ IN}$ represents the corresponding content in the intestinal fraction before dialysis.

2.8. Toxicity in Artemia salina

A toxicity assay was conducted using the *Artemia salina* model, following the protocol of Banti and Hadjikakou [20], with modifications [21]. The *A. salina* cysts (Cystemia, ARTEMIAS S. de R.L. de C.V., Hermosillo, Sonora, Mexico) were hatched according to the supplier's specifications. A 24-well plate was used, and 1900 µL of artificial seawater and 10 active nauplii were added to each well. The dry extract was dissolved in artificial seawater, and serial dilutions were prepared. Each concentration was tested in triplicate. Subsequently, 100 µL of the extract was added at different concentrations (50, 100, 200, 500, and 1000 µg/mL, all prepared in artificial seawater). Artificial seawater was used as a negative control and Sodium Dodecyl Sulfate (SDS) as a positive control exclusively to validate the viability and sensitivity of the assay. The plates were incubated for 24 h at 25 °C in the dark. The nauplii were then examined individually under a stereomicroscope (Leica Zoom 2000, Wetzlar, Germany), and those that showed no movement during 10 s of observation were considered dead. The mortality rate was calculated as the following Equation (6):

$$Mortality\ (\%) = \left(\frac{N_t - N_l}{N_t}\right) \times 100, \quad (6)$$

where N_t is the total number of nauplii at the start of the experiment and N_l is the number of live nauplii at the end of the exposure period.

Artemia salina is widely used as an alternative model for preliminary toxicological studies due to its ease of handling, short life cycle, and sensitivity to bioactive compounds. Its simple nervous system lacks the structures associated with pain perception and emotionality in higher organisms; therefore, its use generally does not require approval from animal ethics committees [22].

2.9. Statistical Analysis

All experiments were performed in triplicate (n = 3), and results are presented as mean ± standard deviation and were subjected to analysis of variance (ANOVA) and

comparison of means using Tukey's test, with OriginLab2023® software (version 2023; OriginLab Corporation, Northampton, MA, USA), at a significance level of $p < 0.05$. Mortality data were transformed into Probit units and analyzed using linear regression against the logarithm of the concentrations to estimate the median lethal concentration (LC_{50}) and its 95% confidence interval in Microsoft Excel.

3. Results and Discussion

3.1. Phytochemical Characterization

The results of the phytochemical characterization are shown in Figure 1. The coffee pulp extract had a total polyphenol content of 90.33 ± 8.16 mg GAE/g of extract, a total flavonoid content of 60.48 ± 4.33 mg CE/g of extract, and a condensed tannin content of 27.27 ± 0.39 mg PC1eq/g of extract. Statistical analysis revealed significant differences among the phytochemical assays, with the highest values obtained for total polyphenol content, followed by total flavonoid content and condensed tannin content. Flavonoids constitute a subclass of polyphenolic compounds, and both the Folin–Ciocalteu and $AlCl_3/NaNO_2$ colorimetric methods present limited specificity: the Folin–Ciocalteu reagent can react with other reducing substances present in the matrix [23], while the $AlCl_3/NaNO_2$ method, although relatively specific for flavonols, flavones, and catechins, also shows considerable reactivity toward phenolic acids [24]. The HCl-butanol assay, in contrast, relies on the acid-catalyzed oxidative depolymerization of proanthocyanidins to anthocyanidins under heat [25], a mechanism specific to condensed tannin-type structures.

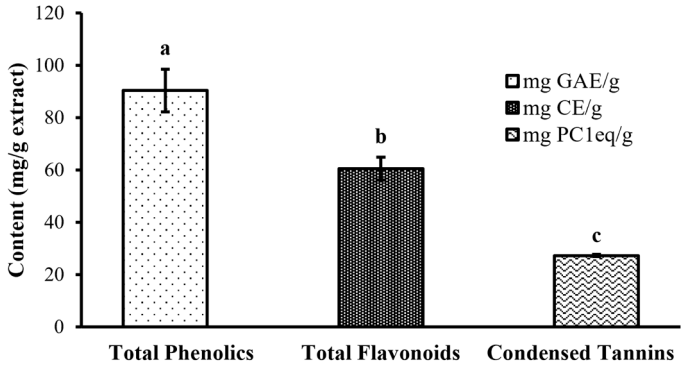

Figure 1. Phytochemical characterization of coffee pulp extract (*Coffea arabica*). Different letters indicate significant differences ($n = 3$; Tukey, $p < 0.05$).

The polyphenol content was similar to that reported by Kusumocahyo et al. [26], using coffee pulp and 50% ethanol, obtaining values of 96.35 mg GAE/g of extract, as well as Silva et al. [27], using coffee husks (*Coffea arabica*), which reported values of 97.89 mg GAE/g of extract. Similarly, Dos Santos et al. [28] reported a polyphenol content of 79.53 mg GAE/g of coffee pulp extract (*Coffea robusta*). Other studies using coffee pulp have reported values of 20.86 and 46.72 mg GAE/g dry weight, although the species used was *Coffea robusta* [29]. Finally, it should be noted that gallic acid is not necessarily the predominant phenolic compound in coffee pulp [30], which should be considered when interpreting the results.

For flavonoid content, higher values have been reported, such as 70.73 mg CE/g of coffee pulp extract [31], 27.22 mg CE/g of coffee pulp extract (*Coffea arabica*) [32], as well as values of 15.69 mg CE/g of coffee husk extract (*Coffea arabica*), all of which are lower than those obtained in the present study. Additionally, values of 18.77 and 36.79 mg CE/g dry weight of coffee pulp (*Coffea robusta*) have been reported [29], indicating that the flavonoid content falls within the range of values reported in the literature.

Regarding condensed tannins, a study conducted by Bamikale et al. [33] reported a content of 60.88 mg procyanidins/g of coffee pulp extract (*Coffea arabica*) using a hybrid

microwave-ultrasound technology and 70% acetone. These results were higher than those obtained in the present study. The differences observed in the phytochemical characterization may be related to the *Coffea* species, the characteristics of the raw material, and the extraction conditions [33–35]. Alkaline and acidic conditions have also been shown to extract phenolic compounds and tannins from coffee pulp [36,37], although the more aggressive conditions required may be less favorable for food or nutraceutical applications compared to acetone extraction. Taken together, these results demonstrate the presence of significant amounts of phenolic compounds in the coffee pulp extract, with polyphenols and flavonoids predominating.

3.2. Antioxidant Activity

Table 1 shows the results of the antioxidant capacity of the coffee pulp extract as determined by the DPPH, ABTS, TAA, and FRAP assays. The highest activity was reported in the TAA assay (325.11 ± 7.40 mg TE/g of extract), which was statistically significant compared to the other assays. On the other hand, there was no significant difference between FRAP (106.03 ± 9.35 mg TE/g of extract) and DPPH (105.41 ± 8.18 mg TE/g of extract). Finally, ABTS (69.41 ± 2.63 mg TE/g of extract) had the lowest antioxidant potential and was statistically different from TAA, FRAP, and DPPH.

Table 1. Antioxidant capacity of coffee pulp extract (*Coffea arabica*) in different experiments.

Sample	Antioxidant Assay (mg TE/g Extract)			
	DPPH	**ABTS**	**TAA**	**FRAP**
Coffee pulp extract	105.41 ± 8.18 ^{b}	69.41 ± 2.63 ^{c}	325.11 ± 7.40 ^{a}	106.03 ± 9.35 ^{b}

Different letters indicate significant differences (n = 3; Tukey, $p < 0.05$).

Only one study was found that evaluated TAA in coffee pulp extract (*Coffea canephora*), obtaining values of 11.15 g of ascorbic acid equivalents (AAE)/100 g [38]. However, the comparison is limited in this study due to the use of Trolox as the standard. For FRAP, studies have only been conducted on coffee pulp but not on extracts. The values reported in the literature range from 35.85 to 84.73 mg TE/g of dry weight of coffee pulp (*Coffea robusta*) [29] and 80 mg TEAC/g dry weight for coffee pulp of the *Coffea arabica* species [39]. Interestingly, these values are close to those reported in the present study. Similarly, for DPPH, there are no studies on extracts, but for dry-weight material, values of 5.20–11.02 mg TE/g dry weight have been reported for coffee pulp (*Coffea robusta*) [29] and 21.49 mg TE/g dry weight for *Coffea arabica* [40]. Finally, values of 102.3 and 182.3 mg TE/g have been reported for ABTS in coffee pulp extracts (*Coffea arabica*) [41,42]. These values are higher than those in the present study, although coffee husk (*Coffea arabica*) extracts have also shown values of 22.79 mg TE/g of extract [43], meaning that the results obtained fall within the range of reported antioxidant activities. The observed differences may be due to a variety of factors, such as the coffee species, climatic conditions, phytochemical composition, sample processing, extraction conditions, as well as differences among the analytical methods used [29,44,45]. DPPH, ABTS, FRAP, and TAA assays were used in combination with previous studies on coffee pulp and related phenolic-rich matrices [29,46]. DPPH and ABTS assess radical neutralization mainly through hydrogen atom transfer (HAT) combined with single electron transfer (SET) [47]; the DPPH radical is soluble only in organic media, whereas the ABTS radical cation is soluble in both aqueous and organic media, allowing it to screen both hydrophilic and lipophilic compounds [48]. On the other hand, FRAP evaluates the reduction of Fe^{3+} to Fe^{2+} via the SET mechanism [47]; in its conventional form, this assay requires specific solvent modifications to measure lipophilic antioxidants [48]. The TAA assay is based on the reduction of molybdenum

Mo(VI) to Mo(V) by the sample and the subsequent formation of a green phosphate/Mo(V) complex [46] and was originally developed and validated for the determination of a lipid-soluble antioxidant [13,49]. Given these mechanistic and solubility differences, no single assay alone can fully capture the antioxidant capacity of a complex phytochemical matrix such as coffee pulp extract, and their combined use provides a more comprehensive assessment of its antioxidant potential.

3.3. Inhibition of Carbohydrate-Hydrolyzing Enzymes

The results of the α-amylase and α-glucosidase enzyme inhibition assays for the coffee pulp extract and the positive control (acarbose) are shown in Figure 2. Under the experimental conditions used and at a concentration of 1000 µg/mL, the coffee pulp extract inhibited α-amylase activity by 20.67 ± 2.28%, which was significantly lower than that obtained for acarbose (89.77 ± 0.94%) at the same concentration (Figure 2a). On the other hand, the extract exhibited α-glucosidase inhibitory activity of 34.69 ± 3.80%, which was significantly higher than that obtained for acarbose (10.57 ± 0.98%) when evaluated at the same concentrations (Figure 2b).

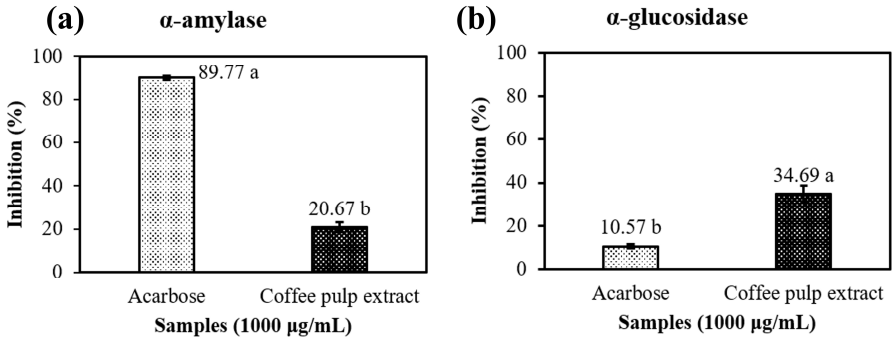

Figure 2. Inhibition of carbohydrate-hydrolyzing enzymes by coffee pulp extract. (**a**) α-amylase, (**b**) α-glucosidase. Different letters indicate statistically significant differences between acarbose and coffee pulp extract within each panel ($n = 3$; Tukey, $p < 0.05$).

Inhibition of α-amylase and α-glucosidase may help slow the digestion of carbohydrates and the release of glucose into the bloodstream after meals. α-Amylase initiates the hydrolysis of dietary starch into oligosaccharides and smaller carbohydrates, whereas α-glucosidase catalyzes the final hydrolysis of these products into absorbable glucose at the intestinal brush border [16,50]. Therefore, simultaneous inhibition of both enzymes may provide a complementary approach to delaying carbohydrate digestion and postprandial glucose release [16,51–53]. Acarbose is one of the most widely used drugs for reducing postprandial glucose levels, but it has adverse gastrointestinal effects [54]. For this reason, researchers have explored alternatives among natural enzyme inhibitors that maintain efficacy but have few or no adverse effects. Coffee pulp extract showed low inhibition values compared to acarbose, and these results are similar to those of previous studies on coffee pulp extracts (*Coffea arabica*), in which α-amylase inhibition was approximately 20% due to the presence of phenolic compounds and flavonoids [32].

In the α-glucosidase assay, the coffee pulp extract exhibited greater inhibitory activity than acarbose; similar results have been reported by Tran and Duong, [55]. However, it is important to note that the α-glucosidase used in this study was derived from *Saccharomyces cerevisiae*, and acarbose has been shown to have lower inhibitory activity against yeast-derived enzymes than against mammalian-derived enzymes in these studies [56]. Therefore, the extract does not necessarily have greater inhibitory activity than acarbose. Additionally, other studies have demonstrated the potential of coffee pulp against both enzymes [50–52,57,58].

On the other hand, the extract showed greater inhibitory activity against α-glucosidase compared to α-amylase, which could be related to the phenolic compounds' higher affinity for that enzyme. This same trend was reported by Dong et al. [53] for coffee grounds. This selectivity towards α-glucosidase may also reflect differences in binding patterns at the enzyme active sites: molecular docking studies on phenolic compounds from coffee by-products show effective binding to key catalytic residues of α-glucosidase, whereas equivalent residues in α-amylase are more frequently bound at inactive sites [53]. Regarding the higher α-glucosidase inhibitory activity of the coffee pulp extract compared with acarbose, phenolic compounds have been reported to act as non-competitive or mixed-type inhibitors [16]. This pattern is consistent with a previous report of coffee pulp extract, which similarly showed higher α-glucosidase inhibitory activity than acarbose [55], and has also been documented for polyphenol-rich extract from other plant matrices against yeast α-glucosidase [56].

These results suggest that coffee pulp has the ability to inhibit enzymes involved in carbohydrate digestion, thereby exhibiting significant antidiabetic potential. Furthermore, this potential may involve additional mechanisms, including antioxidant activity and the modulation of glucose uptake [40,58,59].

3.4. In Vitro Gastrointestinal Digestion

The results of the in vitro digestion assay are shown in Figure 3. The total polyphenol content decreased progressively throughout the simulated digestion phases, from 43.17 ± 1.57 mg GAE/g of extract in the initial extract to 38.59 ± 0.80 mg GAE/g of extract after the oral phase, although this difference was not statistically significant. The content of total polyphenols then decreased significantly during the gastric (31.30 ± 2.30 mg GAE/g of extract) and intestinal (15.99 ± 0.83 mg GAE/g of extract) phases. In the dialysis phase, only 1.56 ± 0.30 mg GAE/g of extract remained in the non-dialyzable fraction (IN), and no polyphenols (N.D.) were detected in the dialyzable fraction (OUT) (Figure 3a).

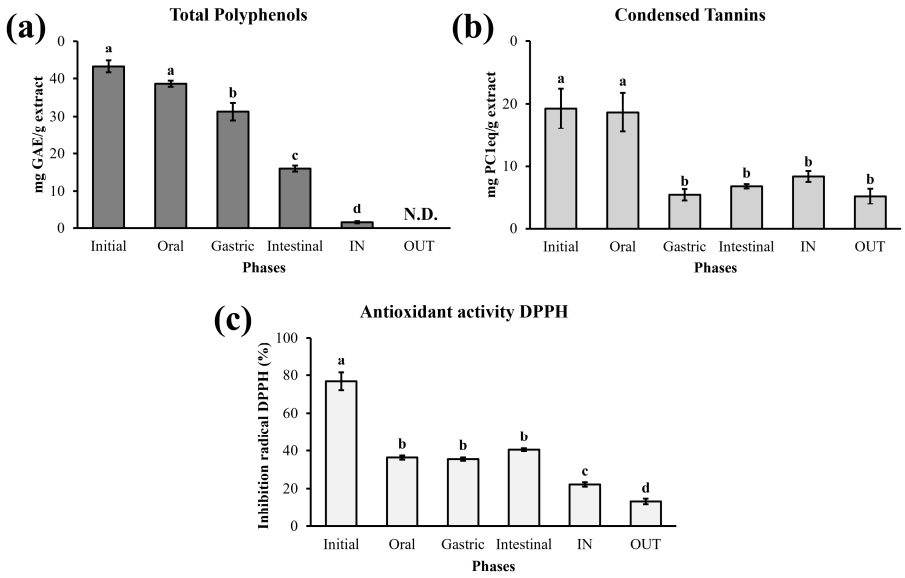

Figure 3. In vitro gastrointestinal digestion of coffee pulp extract. (**a**) Total polyphenols, (**b**) Condensed tannins, (**c**) DPPH antioxidant activity. Different letters indicate significant differences ($n = 3$; Tukey, $p < 0.05$). Values below the limit of quantification were reported as not detected (N.D.).

On the other hand, condensed tannins remained stable during the initial phase (19.24 ± 3.12 mg PC1eq/g of extract) and the oral phase (18.64 ± 3.10 mg PC1eq/g of extract), with no significant difference. These concentrations decreased significantly dur-

ing the gastric phase (5.49 ± 0.92 mg PC1eq/g of extract), with no additional significant changes observed in the intestinal phase (6.82 ± 0.35 mg PC1eq/g of extract) or in the IN (8.39 ± 0.88 mg PC1eq/g of extract) and OUT (5.21 ± 1.22 mg PC1eq/g of extract) dialysis phases (Figure 3b).

Finally, under the conditions evaluated, DPPH antioxidant activity showed a high inhibition percentage in the initial phase (76.88 ± 4.69%), which decreased significantly in the oral phase (36.32 ± 1.12%), gastric phase (35.52 ± 0.90%), and intestinal phase (40.46 ± 0.67%), with no significant difference among the three phases. In the dialysis phase, the IN fraction exhibited 22.16 ± 1.13% inhibition of the DPPH radical, while the OUT fraction exhibited 13.26 ± 1.49% inhibition, both fractions showing statistically significant differences from each other and from the other phases (Figure 3c).

The lower content of total polyphenols and condensed tannins observed in the initial phase of the in vitro digestion assay, compared to the phytochemical characterization (Section 3.1), could be attributed to the different solvents used (distilled water versus 50% ethanol, respectively). The lower solubility of phenolic compounds in aqueous media may reduce the response of the Folin–Ciocalteu assay [60], while the presence of water may also affect the quantification of condensed tannins using HCl-butanol [61]. This pattern was observed for both parameters, although the reduction was proportionally greater for total polyphenols.

Gastrointestinal digestion resulted in a reduction in the phenolic compounds of the coffee pulp extract, which could indicate limited stability. Total polyphenols showed a 63% reduction at the end of intestinal digestion. This decreasing trend is consistent with the reduction values of 43% and 76% estimated from values reported by Yu et al. [52] and Oliveira et al. [58] for soluble dietary fiber from coffee pulp and coffee pulp extract, respectively. However, these results differ from the high stability reported by Khochapong et al. [62], which found a 7.9% reduction for coffee pulp extract. Meanwhile, condensed tannins showed a 65% reduction, similar to that observed for total polyphenols. These values are consistent with those observed by Vázquez-Nuñez [63], where procyanidins (condensed tannins) from coffee pulp had a recovery rate of 19.80% at the end of digestion, representing an 80% reduction. This reduction may be due to the strong interaction of tannins with the components of each digestive phase, as well as to polymerization, depolymerization, or autooxidation when exposed to specific pH conditions [64–67].

In addition, the results obtained are consistent with those observed by Khochapong et al. [62], when an aqueous extract of coffee pulp (*Coffea arabica*) was subjected to in vitro digestion. A reduction in polyphenols, chlorogenic acid, and caffeine was observed, which was associated with a decrease in the extract's antioxidant activity and antimicrobial capacity. However, other studies on coffee pulp and coffee pulp extracts have shown varying changes—including increases, decreases, or constant levels—in phenolic compounds or their activities during the digestion process [30,52,58,62,68]. In fact, it has been found that this behavior varies depending on the matrix, whether it is coffee pulp flour or coffee pulp extracts. Furthermore, these changes may be due to the release of the compounds from the plant matrix or their biotransformation, which is related to their structure, the gastrointestinal environment, and the specificity of the analytical methods used [30,52,68].

On the other hand, the decrease in antioxidant activity is related to the reduction in phenolic compounds, since these are responsible for neutralizing free radicals [69]. However, the decrease does not depend solely on the amount of these compounds, because it has been observed that this trend varies depending on the matrix used. Furthermore, several factors related to changes in phenolic compounds may contribute to this behavior of antioxidant capacity [52,58,62,68].

The gastrointestinal bioaccessibility index (BI_{GI}) showed that 37.04% of the initial total polyphenol content and 35.43% of the initial condensed tannin content remained after the intestinal digestion phase. In contrast, in the dialyzable fraction (DF), no total polyphenols were detected in the dialysate, while condensed tannins showed a DF of 76.39%.

The BI_{GI} values were similar for polyphenols and condensed tannins. These results indicate that a significant proportion of both compounds remain available after digestion, although a considerable fraction was lost or transformed. Oliveira et al. [58] reported BI_{GI} values for total polyphenols of 57% in a coffee pulp extract, while the same extract encapsulated had a value of 71%, highlighting the use of protection strategies to increase bioaccessibility. In addition, values of 24% and 90% have been estimated based on data reported by Yu et al. [52] and Khochapong et al. [62] for soluble dietary fiber from coffee pulp and coffee pulp extract, respectively. For condensed tannins, a BI_{GI} of 15% has been estimated for coffee pulp extract based on data reported by Vázquez-Nuñez et al. [63]. These results demonstrate that, in general, coffee pulp extracts have variable bioaccessibility, which is influenced by the same factors discussed for polyphenol content and antioxidant activity, related to the matrix, biotransformation, and analytical method [52,58,62,68].

For the dialyzable fraction (DF)—also reported as a percentage of bioavailability in the literature—no values could be reported for total polyphenols, since the content of the OUT fraction was lower than the detection limit of the method. This non-detection may be partly attributable to the 100-fold dilution applied during dialysis (2 mL of intestinal digest into 200 mL of buffer), as well as to the reduction of phenolic recovery associated with pH changes and the presence of bile salts during digestion, which can promote precipitation of phenolic compounds and limit the ability of the colorimetric method to accurately quantify them [70]. Furthermore, to the best of our knowledge, no values for total polyphenols have been reported for coffee pulp. Some studies have reported bioavailability values of 15–89% for nuts and seeds [71]. Therefore, total polyphenols exhibit low bioavailability under the conditions used. In contrast, condensed tannins had a DF value of 76.39%, indicating a higher potentially absorbable fraction. This finding may be related to the relative stability of some proanthocyanidins under gastrointestinal conditions [63,72,73]. In fact, based on the content reported by Vázquez-Nuñez et al. [63] for a 24 h dialysis of coffee pulp extract, bioavailability values of 35% have been estimated, while they report an absorption rate of 60% at 48 h. Therefore, the results show a trend similar to that of the present study. Nevertheless, although total polyphenols were not quantifiable in the dialyzable fraction and a portion of the condensed tannins remained in the OUT fraction, this does not mean that they cannot be used, since these compounds can reach the colon and be transformed by the gut microbiota into potentially bioactive metabolites [30,68].

Furthermore, digestion can reduce biological activities, such as antioxidant and antimicrobial activity [71], but it has been shown that encapsulating coffee pulp extracts can preserve biological activity—such as α-amylase inhibition—as well as improve stability and bioaccessibility [58,63]. This means that these protective processes must be taken into account to preserve the activity of the compounds present in the coffee pulp extract.

3.5. Toxicity in Artemia salina

The coffee pulp extract showed very low toxicity against *A. salina*, causing a maximum mortality rate of 10% at the highest concentration tested (1000 µg/mL), compared with the 5% mortality in the negative control. Although a Probit analysis had been planned, this mortality response was insufficient to produce an adequate estimate of the effect or a reliable mathematical fit, since the 50% mortality threshold was not reached. Consequently, following standard toxicological criteria [74,75], the median lethal concentration (LC_{50}) was reported as >1000 µg/mL after 24 h of exposure, indicating low toxicity under

the conditions evaluated according to the toxicity index of Meyer and Clarkson, which consider values > 1000 µg/mL as non-toxic [76]. This result differs from that reported by Marin-Tello et al. [77], who evaluated an aqueous coffee pulp extract (*Coffea arabica*) on *Artemia franciscana*, finding an LC_{50} of 173.53 µg/mL and classifying it as moderately toxic. Furthermore, that same study evaluated the toxicity of an extract of roasted coffee grounds (LC_{50}: >1000 µg/mL), which was considered relatively harmless. Another study by Purnamasari et al. [78] evaluated an ethanolic extract of coffee leaves (*Coffea canephora*) on *A. salina*, obtaining an LC_{50} of 139.378 ppm, which was considered relatively toxic. The differences compared to the present study may be due to the *Artemia* species used in the study by Marin-Tello et al. [77], as well as the sample processing. Additionally, it is interesting to note the behavior observed in coffee pulp extract, roasted coffee grounds, and coffee leaves, which exhibit toxicity levels ranging from safe to relatively toxic, despite being derived from plant matrices, demonstrating the importance of preliminary toxicity studies to ensure the safety of the extract.

The low toxicity observed in *Artemia salina* is consistent with in vivo studies in mice [79], in which acute oral administration of coffee pulp at 2000 mg/kg did not result in mortality or histological changes in the liver after 14 days. Similarly, subchronic oral administration of coffee pulp and coffee pulp extract at 1000 mg/kg for 90 days did not produce any significant effects on body weight, liver or kidney function, or the histopathology of vital organs [80]. Furthermore, clinical studies in healthy volunteers have reported that consumption of up to 28 g/day of coffee pulp juice concentrate for 12 weeks did not result in adverse events or significant changes in hematological, hepatic, renal, or cardiac parameters [81,82]. Taken together, these results and the LC_{50} value from the present study provide evidence of the low toxic potential of coffee pulp-derived products and support their potential for future health and food applications.

4. Limitations

This study has some limitations. The phytochemical characterization was based on colorimetric methods; therefore, the results represent overall estimates and do not allow for the specific identification or quantification of individual phenolic compounds. The complementary use of techniques such as liquid chromatography with ultraviolet detection (LC-UV) or coupled with mass spectrometry (LC-MS) would allow for a more detailed characterization of the phenolic profile. Furthermore, the analysis was limited to the soluble phenolic fraction, without evaluating bound compounds. On the other hand, no enzymatic kinetic analysis was performed to determine the specific mode of inhibition of the extract against carbohydrate-hydrolyzing enzymes. Addressing these aspects would allow for a more complete characterization of coffee pulp-derived extracts in future studies.

5. Conclusions

The coffee pulp extract exhibited a phytochemical profile characterized by the presence of polyphenols, flavonoids, and condensed tannins, as well as high antioxidant capacity. In addition, the extract showed a moderate ability to inhibit enzymes involved in carbohydrate digestion, particularly α-glucosidase, which could suggest an affinity of the compounds for this enzyme. On the other hand, in vitro digestion demonstrated the low stability of the extract, because the levels of the compounds, as well as the antioxidant activity, decreased, and only the condensed tannins were quantifiable in the dialyzable fraction, meaning that most of the compounds could be used mainly by the gut microbiota. For this reason, encapsulation systems show promise for maintaining the stability and bioactivity of coffee pulp extracts. Finally, the preliminary safety assessment classified the extract as non-toxic. These results highlight the bioactive potential of coffee pulp, as well as the limitations

on its use, which may facilitate the development of coffee pulp-based products, such as nutraceuticals, thereby adding value to this byproduct of the coffee industry.

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Institutional Review Board Statement: Ethical review was not required for this study, as *Artemia salina* is an invertebrate species not classified as a protected animal under international ethical guidelines for animal experimentation (e.g., EU Directive 2010/63/EU). Nonetheless, the assay was conducted following recognized laboratory safety and ethical standards.

Informed Consent Statement: Not applicable.

Data Availability Statement: The data supporting the findings of this study are available within the article. For more information about the datasets and experimental protocols please contact the corresponding author.

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Abbreviations

The following abbreviations are used in this manuscript:

AAE	Ascorbic Acid Equivalents
ABTS	2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid
ANOVA	Analysis of variance
BI_{GI}	Gastrointestinal Bioaccessibility Index
CE	Catechin Equivalent
CT	Condensed Tannins
DF	Dialyzable Fraction
DNS	3,5-dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
FRAP	Ferric Reducing Antioxidant Power
GAE	Gallic Acid Equivalent
LC_{50}	Median lethal concentration
N.D.	Not Detected
PC1eq	Procyanidin C1 Equivalent
pNPG	4-nitrophenyl β-D-glucopyranoside
SDS	Sodium Dodecyl Sulfate
TAA	Total Antioxidant Activity
TE	Trolox Equivalent
TFC	Total Flavonoid Content
TPC	Total Polyphenol Content
TPTZ	2,4,6-tris(2′-pyridyl)-1,3,5-triazine

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