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Antibacterial potential of the ethanolic extract of pericarp of cacao (*Theobroma cacao* L.) on *Staphylococcus aureus* and *Escherichia coli*

Potencial antibacteriano del extracto etanólico de pericarpio de cacao (*Theobroma cacao* L.) sobre *Staphylococcus aureus* y *Escherichia coli*

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Abstract

Cocoa pericarp is a waste product in the cocoa industry, but it contains a large amount of bioactive compounds with high antibacterial potential. The objective of this research was to evaluate the antibacterial potential of the ethanolic extract of cocoa pericarp (*Theobroma cacao* L.) on *Staphylococcus aureus* and *Escherichia coli*. The ethanolic extraction was carried out by digestion was performed (Soto Hernández et al., 2019). Four (4) concentrations (25 mg/mL, 50 mg/mL, 75 mg/mL, and 100 mg/mL) and a negative control (sterilized distilled water) were established. The agar well diffusion method was used (Balouiri et al., 2016). The results showed that *S. aureus* bacteria were much more sensitive to cocoa pericarp ethanolic extract (CPE) than *E. coli*, yielding average inhibition zones of 10.8 and 13.53 mm at concentrations of 50 mg/mL and 75 mg/mL, unlike E. coli, which had averages of 9.88 and 9.46 mm at the same concentrations. The concentration with the greatest inhibitory power on both bacteria was 100 mg/mL, yielding average inhibition halo values of 14.84 and 17.68 mm on *E. coli* and *S. aureus*, respectively. These results demonstrate that EPC has high antibacterial power on Gram-positive and Gram-negative bacteria, with the former being more susceptible to this natural antimicrobial. In view of the antibacterial properties observed, EPC has the potential to be developed as a natural antimicrobial agent in food preservation.

Keywords: ethanolic extraction, cocoa pericarp, natural antibacterial.

Resumen

El pericarpio de cacao constituye un producto de desecho en la industria del cacao, sin embargo, éste posee una gran cantidad de compuestos bioactivos de alto potencial antibacteriano. El objetivo de esta investigación fue evaluar el potencial antibacteriano del extracto etanólico del pericarpio de cacao (Theobroma cacao L.) sobre Staphylococcus aureus y Escherichia coli. La extracción etanólica se realizó por digestión (Soto Hernández et al., 2019). Se establecieron cuatro (4) concentraciones (25 mg/mL, 50 mg/mL, 75 mg/mL, y 100 mg/mL) y un control negativo (agua destilada esterilizada). Se utilizó el método de pocillo o Agar well diffusion method (Balouiri et al., 2016). Los resultados mostraron que la bacteria S. aureus fue mucho más sensible al extracto etanólico de pericarpio de cacao (EPC) que E. coli, arrojando promedios de halos de inhibición de 10,8 y 13,53 mm a la concentración de 50 mg/mL y 75 mg/ml, a diferencia de E. coli con promedios de 9,88 y 9,46 mm a las mismas concentraciones. La concentración con mayor poder inhibitorio sobre ambas bacterias fue aquella de 100 mg/mL, arrojando valores promedios de halos de inhibición de 14,84 y 17,68 mm sobre E. coli y S. aureus respectivamente. Estos resultados demuestran que el EPC posee un alto poder antibacteriano sobre bacterias Gram positivas y Gram negativas, siendo las primeras más susceptibles a este antimicrobiano natural. En vista de las propiedades antibacterianas observadas, el EPC tiene el potencial de desarrollarse como un agente antimicrobiano natural en la conservación de alimentos.

Palabras clave: *extracción etanólica, pericarpio de cacao, antibacteriano natural.*

1 Introduction

Part of the stability and safety of food products is based primarily on preservation through the addition of natural antimicrobial agents. This is because the use of synthetic antimicrobial agents has caused problems in humans (allergic reactions, migraines, degenerative diseases, various types of cancer, among others). which is why there is a need to find preservation alternatives that offer the same antimicrobial properties and food compatibility (Kumar et al., 2025; El Sayed et al., 2022; Rodríguez and Nereyda, 2011).

Some natural antimicrobials are obtained mainly from herbs, plants, and spices, which have been used for decades to increase the shelf life of food. There are several reports in the literature on the antimicrobial activity of spices, herbs, and plants or their extracts (Khan et al., 2025; Guerrero et al., 2021; Solis, 2011; López et al., 2013; Ludy et al., 2013). Among the main bioactive compounds extracted from plants and seeds are terpenes, glucosinolates, and phenolic compounds. These, such as flavonols, typically found in fruits and green tea, have antibacterial activity.

Cocoa beans are also a rich source of polyphenols. The total polyphenol content of cocoa is significantly higher than that of acai berries, blueberries, and pomegranates, meaning that its antioxidant activity is much greater than that of other fruits (Crozier et al., 2011). Cocoa is scientifically known as Theobroma cacao L., where the term cocoa comes from the Nahuatl word 'cacahualt'. The name of the genus comes from the Greek 'theos' (God) and 'broma' (food) and means 'food of the gods' (Orwa et al., 2009). The different parts of the fruit (Figure 1) include the seed, mucilage, tegument, shell, endosperm, cotyledons, and pericarp or husk. The latter is the part of the fruit that covers the seed and is obtained by shelling it. This material represents approximately 12% of the seed's weight and is obtained after the roasting process. It is dry, crunchy, and brown in color (Kalvatchev et al., 1998).

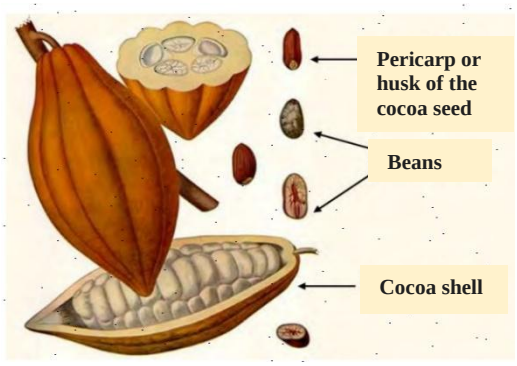

Figure 1. Parts of the cacao fruit (Beckett, 2008).

The cacao bean is a potential source of antibacterial agents and has been used since ancient times by the Aztecs to treat intestinal diseases (Dillinger et al., 2000). The husk or pericarp is usually treated as a food source for animals due to its dietary fiber content, but its alkaloid content restricts its use. Currently, there has been an increase in studies related to this type of waste and its possible use, as it represents an important component of agricultural and agroindustrial waste worldwide, constituting a good source of renewable resources and energy. Internationally, possible uses for cocoa husks are being developed, such as a source of soil fertilizers (Rojo-Poveda et al., 2020; Ayeni, 2010), animal feed for poultry and other animals (Leiva et al. 2022; Alemawor et al., 2009), and a source of pectins and gums (Ortiz et al., 2023; Barazarte et al., 2008). However, there are few studies related to its use as a natural antimicrobial agent, and most of the work refers to other parts of the fruit.

Various studies have evaluated the antimicrobial activity of cocoa on different microorganisms, including *Salmonella*, *Listeria* sp., *Staphylococcus aureus*, and *Escherichia coli*. The latter are highly implicated in foodborne illnesses (FBIs). *Staphylococcus aureus* consists of Gram-positive cocci and is an important bacterium not only because it causes infections in various parts of the human body, but also because it causes foodborne toxic infections (Goudsmit et al., 2021; Borraz, 2006). With regard to poisoning caused by *S. aureus*, it is known that most outbreaks are caused by coagulase-positive *S. aureus*, as very few coagulase-negative strains are capable of producing enterotoxins (staphylococcal food poisoning, SFP). Therefore, it is important to mention that staphylococcal enterotoxins are among the few bacterial toxins of a protein nature that are heat-resistant (Perdomo and Meléndez, 2004). On the other hand, *Escherichia coli* belongs to the Enterobacteriaceae family, the representatives of this species are Gram-negative and oxidase-negative bacilli. Among the pathogenic strains of *E. coli* is *E. coli* O157:H7. This is an enterohemorrhagic strain that causes food poisoning due to the production of verotoxin (Welinder-Olsson and Kaijser, 2005). This bacterium is considered the indicator microorganism par excellence for fecal contamination (Edberg et al., 2000). For this reason, the quantification of *E. coli* in water bodies and food in tropical environments as the main representative of the thermotolerant coliform group has gained importance in the last decade (Romeu et al., 2012).

There are several studies associated with the extraction of bioactive compounds present in plants and fruits, as well as cocoa beans and shells, which have an antimicrobial effect on different microorganisms such as *E. coli*, *Shigella dysenteriae*, *Bacillus cereus*, and *S. aureus* (Llerena et al., 2023; Ariza et al., 2013; Wulandari et al., 2012; Nsor-

Atindana et al., 2012). Most studies used extraction methods with organic solvents or hydroalcoholic extraction, which are fundamental techniques for the analysis of secondary metabolites and polyphenols.

In this regard, given that the pericarp is a waste by-product of the cocoa industry, and in accordance with the antimicrobial precedents of the fruit seed, this study set out to evaluate the antibacterial potential of the ethanolic extract of cocoa pericarp (*Theobroma cacao* L.) on strains of *Staphylococcus aureus* and *Escherichia coli*.

2 Materials and Methods

The study was conducted in the Food Technology Laboratory of the Departmen t of Food Technology, and microbiological analyses were performed in the Microbiology Laboratory of the Department of Biology and Animal Health. Both departments belong to the School of Agricultural and Environmental Sciences of the Universidad de Oriente, Maturín Municipality, Monagas State, Venezuela.

2.1 Obtaining the ethanolic extract

2.1.1 Obtaining the sample

Samples of dry Criollo cocoa pericarp were used, donated by the cocoa processing company CHOCOLATERA VILLROS C.A., located in the municipality of Caripito, Monagas State, Venezuela. These samples comprise a waste by-product of the company.

2.1.2 Sample preparation

Three hundred grams (300g) of pericarp were weighed and ground to a particle size of 0.5 mm (Ariza et al., 2013) using Thomas-Wiley equipment (Model Arthur H., Philadelphia, PA, USA). The sample obtained was weighed and hermetically sealed until the extraction process.

2.2 Preparation of the ethanolic extract

Moisture and fat content were previously determined according to AOAC (1990) to ascertain the characteristics of the pericarp sample. With the help of these results and the bibliographies consulted, the extraction method used in this study was established, which was the digestion extraction method following the methodology described by Soto Hernández et al. (2019). In this case, approximately 15 g of sample (ground pericarp) was first weighed using an Adventuer analytical balance (TM AR2140, NJ), then placed in a 250 mL distillation flask and 60 mL of ethanol was added, at a ratio of 1:4 in relation to the mass of the sample. A water-circulating condenser or Allihn cooling tube was then attached to the top of the flask. The system was then mounted on a CORNING heating plate (Stirrer PC 920, USA) with constant magnetic stirring, and extraction was performed by vacuum filtration in triplicate for approximately 6 hours. After the extraction time had elapsed, filtration was carried out using a porcelain funnel with Whatman No. 1 filter paper and a Gast Manodacturing Inc. vacuum pump (8 DAA-VGSIS-ED. USA.).

2.2.1 Solvent distillation

The filtered extract obtained was placed in a Rotaevaporator flask (R-210, Switzerland) with a Heating Bath (B-491, Switzerland). This flask was weighed once washed and dried to determine the yield of the extract, and the solvent was evaporated at 70°C at a vacuum pressure of 600 mmHg using a vacuum pump (BOECO R-300, Germany). A water chiller was used to regulate the temperature, following the methodology described by Soto Hernández et al. (2019). Once the solvent had been separated from the extract, the flask was weighed and the extract obtained was placed in an amber bottle, which was stored in a freezer at -6°C until use.

2.3 Preparation of concentrations

Once distillation was complete, the different concentrations to be used in the test were determined. Taking into account the research consulted (Wulandari et al., 2012; Sucuzhañay, 2015) and previous experimental tests using cocoa beans and shells, solutions at concentrations of 25 mg/mL, 50 mg/mL, 75 mg/mL, and 100 mg/mL were used as criteria for this study, and distilled water was used as a negative treatment, respectively. The negative control, being an inert solvent, will have no inactivating effect on them.

Four 25 mL volumetric flasks were identified corresponding to each solution of the extract at concentrations of 25 mg/mL, 50 mg/mL, 75 mg/mL, and 100 mg/mL. To do this, a stock solution of cocoa pericarp extract (CPE) with a concentration of 3000 mg/mL was prepared in advance, and from this, the different dilutions were prepared using sterile distilled water as a solvent until the respective concentrations of 25 mg/mL, 50 mg/mL, 75 mg/mL, and 100 mg/mL. The volumes of each flask were then measured with sterile distilled water (as a solvent) using a 10 mL volumetric pipette up to the calibration line.

2.3.1 Determination of concentration (C) by Spectrophotometry

To chemically determine that the dilutions were correct, the mathematical relationship of the straight line and Lambert-

Beer's law (equation 1) were used. For this purpose, a calibration curve was made for the cocoa pericarp extract in ethanol solution, with its respective blank of pure solvent. Once this preliminary step was completed, the samples were passed through the Thermo Electron Corporation Evolucion 300 spectrophotometer at a wavelength of 750 nm (Ordoñez et al., 2019; Iglesias-Guevara et al., 2022). In order to obtain the respective absorbances for the concentration, m was cleared. The concentration data will be shown in Table 2.

$$y = mx + b$$

Where:

y: Is the absorbance measured by the equipment.

x: Is the concentration (what we want to verify).

m: Is the slope (sensitivity of the method).

b: Is the intercept (the "noise" of the target).

2.4 Determination of the Effectiveness of EPC on the Inactivation of S. aureus and E. coli in vitro.

2.4.1 Microbiological Analysis (bacterial inactivation based on the concentrations used in the study)

Microbiological analyses were performed in triplicate for each of the concentrations.

2.4.2 Preparation of the inoculum

The *E. coli* and *S. aureus* strains were obtained from the culture collection of the Microbiology Laboratory of the School of Agricultural and Environmental Sciences of the Universidad de Oriente (UDO), Monagas state, Venezuela. These were kept in slanted tubes with soy trypticase agar (HIMEDIA) at a temperature of 5 °C until use. The inocula were obtained by reactivating the strains in nutrient broth at 37 °C for 24 hours (Ramos et al., 2012).

2.4.3 Preparation of culture medium

A total of 36 nutrient agar plates were prepared and labeled as follows: 18 for *E. coli* and 18 for *S. aureus*. The culture medium was prepared based on the total number of plates to be used, following the methodology described by the COVENIN standard (1292-89), for which approximately 11.76 g of nutrient agar was weighed and diluted in 360 mL of distilled water and sterilized in an autoclave. Once the medium was sterilized, it was tempered to 45°C and added at a rate of 15 mL per plate. Finally, it was left to solidify for use.

2.4.4 Determination of antibacterial potential

The determination of antibacterial activity and comparison of the degree of inhibition of the extract on different bacteria was performed using the well diffusion method proposed by Balouiri et al. (2016), which is widely used to evaluate the antimicrobial activity of plants or microbial extracts (Magaldi et al., 2004).

First, the microorganism was seeded using the streak method on the surface of the previously prepared plate (Ramos et al., 2012). Next, using a sterile punch, three 5 mm diameter holes were made per plate (Figure 2) and a volume of 1 µl of the concentrations of each treatment described in Table 8 was added to each well using an automatic micropipette. The plates were placed in the incubator without inversion for a period of 24 hours at 37°C.

Table 1. Proposed treatments to determine antibacterial potential against *E. coli* and *S. aureus*.

Treatment	**Characteristics**
T_1	Sterilized distilled water
T_2	Ethanolic extract 25 mg/mL
T_3	Ethanolic extract 50 mg/mL
T_4	Ethanolic extract 75 mg/mL
T_5	Ethanolic extract 100 mg/mL

2.4.5 Determination of zones of inhibition

After 24 hours, the zones of inhibition in each well were measured (mm) using a vernier caliper.

2.4.6. Comparison of the degree of inhibition obtained using EPC on the respective bacteria

To compare the inhibitory degree of T5 (100 mg/mL) on *E. coli* and *S. aureus*, a Student's t-test for means with n=18 was used. The best concentration of the ethanolic extract obtained from the previous objective was used.

Figure 2. Representation of the agar plate with the respective wells for measuring inhibition zones.

2.5 Experimental design

A completely randomized design was used with five treatments distributed in distilled water as a negative control, 25 mg/mL, 50 mg/mL, 75 mg/mL, and 100 mg/mL (Table 1). To study the inhibition of the different treatments evaluated, the analyses were performed in triplicate with three replicates for each treatment, for a total of 45 observations per microorganism.

2.5.1 Statistical analysis

The results were analyzed using analysis of variance (ANOVA) to determine significant differences between treatments. P-values were calculated using Tukey's test with a significance level of 5%. The InfoStaf statistical package version 2016 was used for data analysis (Di Rienzo et al., 2017). A comparison of the inhibitory degree of T5 on each microorganism was also performed using a Student's t-test, where the null hypothesis (Ho) established that there is no significant difference in the inhibitory degree of the microorganisms studied, and the alternative hypothesis (Hi) stated that there is a significant difference between them. An n=18 and a significance level of 5% were used.

3 Results and Discussion

3.1 Determination of concentration (C) by Spectrophotometry

When analyzing the experimental data obtained at 750 nm, compliance with Beer-Lambert's Law was observed, evidencing a linear relationship between the concentration of EPC and its respective absorbance (Table 2). When comparing the values, it is noteworthy that the instrumental response factor of absorbance units per ppm indicated good preparation of the dilutions and excellent sensitivity of the spectrophotometer at the selected wavelength. This validates the stability of the ethanolic extract of the pericarp and ensures that the readings are within the linear dynamic range of the equipment, allowing reliable analytical quantification without detector saturation effects.

The values obtained for polyphenol concentration are consistent with the results obtained by Ordoñez et al. (2019), Iglesias-Guevara et al. (2022), and Monroy et al. (2023), who at these wavelengths were able to validate the concentrations obtained from the polar soluble extracts of cocoa seed pericarp, such as epicatechins, catechins, tannins, phenolic acids, anthocyanins, theobromine, and caffeine.Para determinar la efectividad de la luz ultravioleta sobre microorganismos como *Escherichia coli* y *Listeria innocua* inoculados a un nivel de 10^9 y 10^7 UFC/100g, respectivamente en la piña fresca cortada, las piezas fueron tratadas con luz ultravioleta durante 5 min a una distancia de 15 y 8 cm (dosis de 1.479 y 2.064 kJ/m2 para cada tratamiento).

Table 2. Concentration of EPC dilutions by UV-Visible spectrophotometry.

Absorbance (750 nm)	Concentrations
0.176	25 ppm
0.355	50 ppm
0.528	75 ppm
0.702	100 ppm

3.2 Determination of the antimicrobial effect of EPC at different concentrations on the growth of S. aureus and E. coli.

The analysis of variance showed significant differences ($p<0.05$) for treatments T1 (sterilized distilled water), T2 (25 mg/mL), and T5 (100 mg/mL) applied to *E. coli*. Table 3 shows the inhibition halos (mm) obtained with the different treatments used in vitro on *E. coli* and *S. aureus* bacteria. The concentrations of the treatments are 25 mg/mL (T2); 50 mg/mL (T3); 75 mg/mL (T4); 100 mg/mL (T5) and the control (T1). The results for *E. coli* in the case of T1 (control with sterilized distilled water), T2 (25 mg/mL), and T5 (100 mg/mL) showed significant differences ($p<0.05$) between each other, while T3 and T4 with concentrations of 50 mg/mL and 75 mg/mL, respectively, did not show significant differences between their values, yielding inhibition halos of 9.46 mm and 9.88 mm, respectively. Finally, in this case, T5 showed greater effectiveness compared to the other treatments, yielding inhibition halos with a mean of 14.84 mm (Figure 4). On the other hand, T2 showed lower effectiveness, registering an average inhibition halo value of 5.06 mm (Figure 3). This indicated that the inhibition zones increased in diameter over the *E. coli* population as the EPC concentration increased; therefore, the higher the EPC concentration, the greater the inhibition of the bacteria.

In the case of *S. aureus* (Table 3), statistical analysis showed significant differences ($p<0.05$) for the treatments. Inhibition zones began to form with T2 at a concentration of 25 mg/mL with an average diameter of 4.6 mm (Figure 5). Concentrations T3 and T4 showed values of 10.80 mm and 13.53 mm, respectively; however, at the highest concentration of 100 mg/mL (T5), an average diameter of 17.68 mm was obtained (Figure 6). The negative control with sterile distilled water (T1) did not show an inhibition zone. This means that EPC had an antibacterial effect against the growth of this bacterium. In turn, it can be seen that the higher the concentration of EPC, the greater the antibacterial effect, where a larger diameter of the inhibition zone was achieved. It should

be noted that *S. aureus* showed greater sensitivity to the polyphenolic compounds of EPC compared to *E. coli.*

Table 3. The zones of inhibition (mm) obtained with the different treatments used in vitro on *E. coli* and *S. aureus* bacteria (averages ± standard deviation).

	Treatments	*E. coli*	*S. aureus*
T_1	Sterilized distilled water	0,00 ^{d}	0,00 ^{e}
T_2	25 mg/mL	5,06±1,70 ^{c}	4,6±1,22 ^{d}
T_3	50 mg/mL	9,46±1,1 ^{b}	10,8±2,42 ^{c}
T_4	75 mg/mL	9,88±1,79 ^{b}	13,53±2,06 ^{b}
T_5	100 mg/mL	14,84±1,32 ^{a}	17,68±2,20 ^{a}

*Different letters indicate significant differences ($p<0.05$)

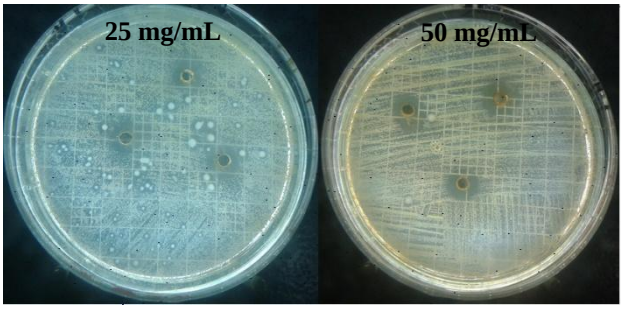

Figure 3. Zones of inhibition (measured in millimeters) of EPC at concentrations of 25 mg/mL and 50 mg/mL using the well diffusion method on *E. coli*.

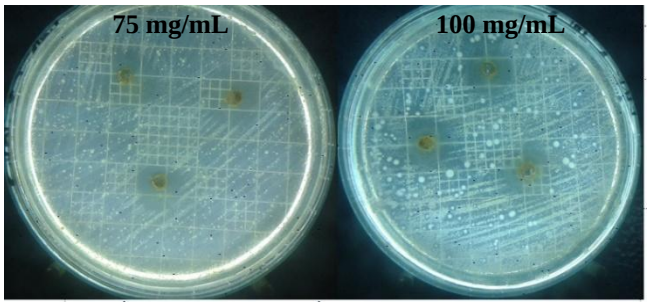

Figure 4. Zones of inhibition of EPC at concentrations of 75 mg/mL and 100 mg/mL using the well diffusion method on *E. coli.*

Larger inhibition halos were observed in *S. aureus* (Gram-positive) than in *E. coli* (Gram-negative) from T3 onwards, showing that *S. aureus* is more sensitive to EPC. This may be due to structural differences in the cell walls of the two bacteria. Gram-positive bacteria have a thick layer of peptidoglycan in their cell wall, while Gram-negative bacteria have a thin layer of peptidoglycan, but one that is much more complex because, in addition to the peptidoglycan layer, it also has an outer lipopolysaccharide membrane (Willey et al., 2023).

In this sense, the antimicrobial effect of EPC is due to the ability of phenolic compounds to penetrate the cell wall, especially in Gram-positive bacteria, because it has a simple structure consisting of an easily hydrolyzable monocomplex, whose cell wall is rigid, unselective, and lacking barrier properties, unlike the thin, multilaminar cell wall of Gram-negative bacteria, which confers a higher degree of resistance (especially due to the outer membrane component at the cell wall level) to antimicrobial agents (Gutierrez et al., 2024; Arlorio et al., 2005).

The antimicrobial capacity of cocoa pericarp extracts lies in a multifunctional chemical offensive led by polyphenols, especially epicatechin and condensed tannins. These compounds act by disrupting the cytoplasmic membrane, where their aromatic rings insert themselves into the lipid bilayer, altering its permeability and causing the loss of vital ions. Simultaneously, the hydroxyl groups of flavonoids exert a chelating effect, sequestering essential metals such as iron (Fe^{+3}) that bacteria require for their enzymatic processes and replication (Ali et al. 2022). In addition, the high affinity of these metabolites for proteins allows the formation of complexes that inactivate extracellular enzymes and transport proteins, collapsing the pathogen's energy metabolism. Together, this molecular attack not only stops cell division (bacteriostatic effect), but at high concentrations such as 100 ppm, it triggers definitive cell lysis, triggering a bactericidal effect.

The antibacterial properties of the ethanolic extract of *Theobroma cacao* L. are attributed to the polyphenols in its composition, including flavonoids, quinones, tannins, catechins, and saponins, which are believed to be responsible for its antibacterial activity. This is because flavonoids, having a variable number of phenolic hydroxyl groups in their chemical structure, easily penetrate the bacterial cell membrane, combine with and precipitate protoplasmic proteins, denaturing them and acting as protoplasmic poisons (Hemeg et al., 2020; Puupponen et al., 2001). Similarly, quinones have a wide range of action, possibly acting on adhesins exposed on the surface of bacteria, as well as on cell wall polypeptides and membrane-bound enzymes. These mechanisms reflect how different natural compounds act on the surface or essential metabolic functions of bacteria to eliminate them. Likewise, tannins can inhibit extracellular microbial enzymes, causing bacterial inactivation through cell adhesion to the surface of cells and enzymes bound to cell membranes and cell wall polypeptides, damaging the cell wall and causing the cell contents to

be emptied, leading to cell death (Arlorio et al., 2005; Akiyama et al., 2001). Catechins can damage the cytoplasmic membrane, causing leaks of important metabolites that inactivate the bacterial enzyme system (Ratnawulan et al., 2025; Volk and Wheeler, 1993). On the other hand, saponins are a group of glycosidic substances that dissolve in water and have the property of forming foam when the solution is shaken.

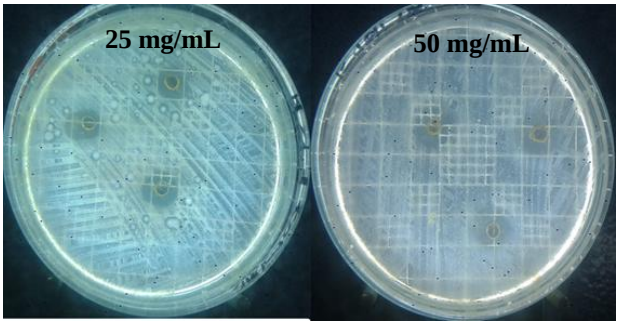

Figure 5. Zones of inhibition (measured in millimeters) of EPC at concentrations of 25 mg/mL and 50 mg/mL using the well diffusion method on *S. aureus*.

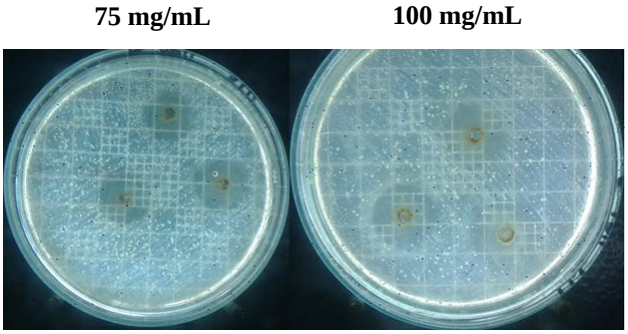

Figure 6. Zones of inhibition (measured in millimeters) of EPC at concentrations of 75 mg/mL and 100 mg/mL using the well diffusion method on *S. aureus*.

All of the above indicates that the compounds present in the extract, the type of microorganism, and the differences in the permeability of bacterial cell walls are the main factors causing microbial inhibition between the two bacteria, explaining why the inhibitory effect is greater in *S. aureus* populations than in *E. coli*.

The results obtained in this study were higher than those achieved by Ariza et al. (2013), who studied the in vitro antibacterial activity of ethanolic extract of cocoa (*Theobroma cacao* L.) against *Escherichia coli*, obtaining zones of inhibition of 1.9 mm with concentrations of 62.5 mg/mL of ethanolic extract of cocoa. For their part, Nsor-atindana et al. (2012) studied the quantification of the total polyphenolic content and antimicrobial activity of cocoa bean shells and reported inhibition halos of 10.98 mm for *S. aureus* at a concentration of 100 mg/mL of ethanolic extract of cocoa husk or pericarp (EPC).

Finally, it should be noted that the bacteriostatic and bactericidal activity of EPC is carried out in several modes of action, including alteration of bacterial cell metabolism, inhibition of cell wall synthesis, alteration of cell membrane permeability, and interference with gene and nucleic acid expression (Percival et al., 2006).

3.3 Comparison of the degree of inhibition obtained using the most effective concentration of EPC on the respective bacteria

Once the treatment with the T5 concentration (100 mg/mL) that caused the greatest inhibitory effect on both microorganisms was obtained, this section finally studied the comparison of the antibacterial effect on each microorganism. When comparing the degree of inhibition of the 100 mg/mL treatment on the *E. coli* and *S. aureus* populations, it was observed that this concentration of EPC is more effective on the latter pathogen, as it reached inhibition halos of 18 mm. The Student's t-test (Table 4) revealed significant differences ($p<0.05$) between the bacteria studied.

S. aureus is a Gram-positive bacterium whose cell wall structure is approximately 25-30 µm thick, single-layered, and consists mainly of peptidoglycan, with a low lipid content (1-4%). These groups of bacteria tend to be more susceptible to the activity of antibacterial components, such as phenolic compounds and penicillin. The simple cell wall structures cause antibacterial compounds to easily enter the cells and find the targets to function (Hemeg et al., 2020; Hawley, 2003). The antibacterial potential of EPC derives from the presence of antibacterial compounds such as tannins and flavonoids (Gutierrez et al., 2024; Volk and Wheeler, 1993). Flavonoids have antibacterial activity through the barrier function of bacterial DNA gyrase, affecting bacterial replication and translation capacity (Gunawan, 2009).

The results reported in this study are higher than those recorded by Sucuzhañay (2015), who studied the antimicrobial effect of aqueous extracts of cocoa shells and seeds (*Theobroma cacao* L.) on strains of *Streptococcus mutans* and reported inhibition halos of 8 to 10 mm using 12.5% and 20% aqueous extracts of cocoa shells on *Streptococcus mutans*. Similarly, Mariani et al. (2010) studied the bacteriostatic effect of cocoa seed extract on the growth of *Streptococcus mutans* in vitro. The authors recorded halos of 6 mm at cocoa concentrations of 0.01% and 8 mm at concentrations of 5%, 15%, and 17.5% against *S. mutans* in aqueous

cocoa seed extracts. Antimicrobial activity may vary depending on the type of microorganism and the content of phenolic compounds present in the extracts, such as polyphenols, flavonoids, epicatechin, and catechin (Percival et al., 2006), associating them with antimicrobial activity as they act specifically against the wall of Gram-positive bacteria, resulting in the loss of cell structure and cell death, altering their structural integrity through hydrophobic and electrostatic interactions with the phospholipid bilayer and porins. This causes membrane stiffening, permeabilization, ATPase inhibition, and efflux pump dysfunction, ultimately leading to cell lysis (Yan et al., 2024; Koech *et al*., 2014).

Table 4. Comparison of susceptibility levels between the bacteria studied (*E. coli* and *S. aureus*).

Microorganisms	Averages (mm)	P
E. coli	17,68	0,0045
	2,83	
S. aureus	14,84	
	1,02	

In the case of *E. coli*, being a Gram-negative bacterium, its resistance to EPC is probably due to the fact that it has a thinner cell wall structure than *S. aureus*. However, it is multilaminar, approximately 10-15 microns thick, with a component that no other group of bacteria currently has. This component is the outer membrane, which is additional to the usual peptidoglycan layer found in most bacteria. It is composed of unique structures called lipopolysaccharides (LPS) that function as endotoxins. It also has an exclusive transport system that is more complex than that found in all cytoplasmic membranes of living beings. This structure provides resistance to detergents, antibiotics (such as penicillin), and toxic agents, serving as a fundamental defensive barrier. The outer membrane consists of three layers: the outer membrane, a thin layer of peptidoglycan on the inside with a high lipid content (11-21%), and the inner membrane. The outer layer consists of two layers of lipopolysaccharide and lipoprotein (Yu et al., 2024; Hawley, 2003). It is possible that the bioactive compounds present in EPC at low concentrations cannot easily penetrate the outer membrane, resulting in less growth inhibition.

Rodríguez et al. (2009) and Alberto et al. (2006) reported in their studies that polyphenolic extracts inhibit Gram-positive bacteria more than Gram-negative bacteria due to the sensitivity of the former to spices and plant extracts. The authors concluded that the greater sensitivity in the case of *Staphylococcus aureus* can be attributed to the structure of its cell wall, which allows antibacterial substances to easily ccess and destroy it, resulting in the release of the cytoplasm.

On the other hand, Sotelo et al. (2010) studied Borojó (*Borojo apatinoi*), a source of polyphenols with antimicrobial activity, and indicated that Borojó extracts exhibited greater inhibition halos for *S. aureus* (Gram-positive) than for *E. coli* (Gram-negative), results that coincide with those obtained by Alberto et al. (2006). These researchers highlight a greater inhibition of extracts with polyphenols in Gram-positive bacteria than in Gram-negative bacteria, due to the sensitivity of Gram-positive bacteria to spices and extracts from herbs, plants, and fruits. Therefore, the greater sensitivity to the antimicrobial effect can be attributed, in the case of *S. aureus*, to the structure of its cell wall (as mentioned above), which allows antibacterial substances to easily destroy the cell membrane, causing the cytoplasm to leak out (Kuwano et al., 2005). The resistance of *E. coli* to antibacterial substances is related to the hydrophilic surface of its outer membrane. This membrane, rich in lipopolysaccharide molecules, acts as a barrier against the penetration of numerous antibiotic molecules and is also associated with enzymes in the periplasmic space, which are capable of breaking down molecules introduced from outside (Yu et al., 2024).

Artiza et al. (2013) reported that ethanol extracts of cocoa (EEC) applied to *E. coli* after a 24-hour exposure period caused cell elongation in the bacteria. At the lowest concentration of 7.8 mg/mL, *E. coli* elongated to 4.286 µm. Cell extension showed cell elongation of 6.859 µm at 15.6 mg/mL, and elongation led to broken cells, indicating permanent disruption and failure to maintain filamentation. This study confirmed that polyphenol causes elongation of *E. coli* cells, as found by Hemaiswarya et al. (2011) and Cui et al. (2012). In turn, they proposed that polyphenols in EEC suppressed the polymerization of ftsZ proteins in the bacterial cytoskeleton, a key role of the protein in septum formation. Inhibition of ftsZ protein activity slows cell division, thereby preventing the bacteria from reproducing by binary fission, and the cell dies.

In general, the response of each microorganism to different concentrations of the extract may be influenced by structural differences between Gram-negative and Gram-positive bacteria, specifically differences in cell wall composition associated with resistance patterns, as well as being affected by variations in the concentrations of phytochemical compounds present in the cocoa pericarp (Gunawan, 2009).

4 Conclusions

Cocoa pericarp, traditionally considered a waste by-product

of the cocoa industry, acted through different mechanisms depending on the structure of the bacterial wall. This study verified the inhibitory differences when faced with *E. coli* and *S. aureus*. T5 (100 mg/mL) showed the greatest antibacterial potential against *S. aureus*, resulting in average inhibition halos of 17.68 mm. This could be attributed mainly to the structural characteristics of the cell wall of this bacterium. The inhibition halos increased in diameter over the population of both bacteria as the concentration of the EPC extract increased.

The satisfactory results obtained in both bacteria confirmed that the extract has a broad-spectrum antimicrobial profile. Its integration as a natural preservative in the food industry can be justified by its multimodal preservation mechanism. Unlike synthetic preservatives, which often have a single target, the bioactive compounds in cocoa can act synergistically. Not only can they inhibit bacterial proliferation by rupturing membranes, but they can also act as powerful antioxidants. This allows for a dual function of microbiological safety and sensory stability. Overcoming the barrier of Gram-negative bacteria is particularly relevant. This expands its use to meat, dairy, and fresh vegetable products, where these pathogens are prevalent. The use of this extract transforms agro-industrial waste into a high-value functional ingredient. As a by-product of the chocolate industry, it is inexpensive to obtain and widely available, meeting circular economy standards. In addition, its natural origin responds to current consumer demand for products free of chemical additives (such as nitrites or sorbates).

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