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Antimicrobial potential of the ethanolic extract of Cadillo (*Bidens pilosa* Linneo.) against *Salmonella typhimurium* causing gastrointestinal infections

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Abstract– ***During a gastrointestinal bacterial infection process, it is common for the population to take antibiotics indiscriminately. However, improper use of these can cause bacterial resistance; for this reason, alternative treatments are sought, using medicinal plants.***

Purpose: To evaluate the antimicrobial potential of the ethanolic extract of Cadillo (Bidens pilosa* L.*) against Salmonella typhimurium, which causes gastrointestinal infections.

Methods: The ethanolic extract was obtained by maceration of 200g. of dried and ground leaves of Bidens pilosa L., in 1250 ml of absolute ethyl alcohol. The presence of the Phytoconstituents was determined by traditional chemical methods. The antibacterial effect was evaluated by the Kirby-Bauer method for concentrations of 125, 250, 500, and 1000 mg/mL. The evaluation of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) was carried out by the Broth Macrodilution method.

Results: A predominance of steroids and terpenoids was found in the extract. The antibacterial activity was present in all extracts, achieving inhibition zones from 10.097 mm ± 0.348 to 33.773 mm ±0.292, with inhibition percentages from 38.9 to 130.0 %. A MIC of 15.6 mg/mL and a MBC of 31.25 mg/mL were obtained.

Conclusions: The ethanolic extract of Bidens pilosa* L. *has a high antimicrobial potential against Salmonella typhimurium, and it is suggested to continue with studies that propose the use of this extract as a new alternative treatment for salmonellosis.

Keywords-- ***L Bidens pilosa* L.*, antibacterial activity, Phytoconstituents, Salmonellosis.***

I. Introduction

The use of medicinal plants has been used since ancient times to cure diseases or alleviate them. This healing form is appreciated for the low cost it generates and the low toxicity rates compared to synthetic products or medications [1,2]. Currently, plants are a great alternative in the health system of developing countries, and it is known that more than 80% of the population in the world uses traditional medicine in the treatment of various diseases, in some cases, it has been the use of medicinal plants in alternative cancer treatment has been reported [3,4].

Bidens pilosa Linnaeus is a plant that is part of the Asteraceae family; In some parts of the world this plant is used as food, while in other countries it is used in traditional medicine to treat various diseases; This plant is distributed globally in tropical and subtropical areas of several Latin American countries; In Peru, this plant is commonly known as "cadillo" or "dry love" and can be found in the department of La Libertad, Huánuco, Ayacucho, Cuzco, Junín, Loreto, San Martin, Amazonas and Cajamarca [4,5]. Various studies have reported that *Bidens pilosa* L. contains a variety of metabolites,

whose pharmacological properties such as antiparasitic, anticonvulsant, antioxidant, antidiabetic, antiulcer, chemopreventive, hepatoprotective, antimicrobial and anti-inflammatory are attributed [6]. Likewise, it has been reported that the consumption of *Bidens pilosa* L. leaves through infusions serves as an antidiabetic, antiemetic, antipyretic and tranquilizer, and also helps combat stomach and urinary tract infections; while the consumption of the flower in infusion is used to combat tonsillitis; while the consumption of the root is indicated to combat headaches [7-9]. Since 2010, studies have been carried out on the effect of aqueous, methanolic, ethanolic, and chloroformic extracts of *Bidens pilosa* L. on Gram-positive and Gram-negative bacteria, especially against the growth of *Pseudomonas aeruginosa, Escherichia coli, Staphylococcus aureus,* and *Candida albicans* [10-12].

The Salmonella genus groups more than 2000 different species according to their antigenic structure characteristics, which is why the species of this genus are identified or formally known by serotypes. Among the known bacteria that cause the highest prevalence of infections in the human body, *Salmonella enterica* is mentioned as causing serious health problems [13-15]. In the last two decades, it has been found more frequently that *Salmonella enterica* serovar Typhimurium (*Salmonella typhimurium*) causes acute infections such as typhoid fever, gastroenteritis, and bacteremia, because this pathogen can colonize the intestinal epithelium and trigger gastrointestinal diseases. In both humans and animals, this type of gastrointestinal infection is generally caused by the consumption of contaminated food or water [16,17]. In the treatment of salmonellosis, multiple drugs have been studied and developed, which are currently no longer effective because Salmonella is creating resistance against antibiotics such as chloramphenicol, sulfonamides, tetracycline, and third-generation cephalosporins used in the treatment of invasive salmonellosis [18,19].

At the end of 2022, the European Food Safety Authority (EFSA) published a community report on zoonoses in the European Union, which indicates that salmonellosis is the second most reported zoonosis in humans (registering 60,050 cases between 2017 and 2022). While, in the United States, *Salmonella typhimurium* is among the 20 most common serovars that cause salmonellosis in humans [20,21]. In Peru, at the beginning of 2001, an in-hospital outbreak due to *Salmonella typhimurium*, which produces extended-spectrum beta-lactamase ESBL SHV-5, was reported [22]. In 2010, an unusual increase in Salmonella cases was detected in isolates from people, mostly pediatric patients from various hospitals in Lima [23]. In 2017 study where molecular typing was carried out on *Salmonella enterica* cultures causing bacteremia in patients in nine public hospitals in Lima-Peru it was reported that the most frequent serovars are Enteritidis (45%), Typhimurium (36%), and Typhi (11%) [24].

Having as a background that *Salmonella enterica* serovar Typhimurium is one of the bacteria that can cause salmonellosis in humans, causing acute infections such as typhoid fever, gastroenteritis, and bacteremia; where the population with the highest prevalence of contracting this disease are children under five years of age and over sixteen years of age; and that since 2000, limitations in antimicrobial therapy have been evident, especially in strains that have been developing resistance to antimicrobials; is that the present research work evaluates the antimicrobial potential of the ethanolic extract of "cadillo" (*Bidens pilosa* L.) against *Salmonella typhimurium* that causes gastrointestinal infections and proposes the use of the extract as a new alternative for the treatment of salmonellosis, taking into account that obtaining Ethanolic extract is one of the most basic processes concerning obtaining organic extracts and essential oils.

II. Material and Methods

The leaf samples of *Bindes pilosa* L. were collected in August 2021 from the Polvorín population center, Chepén district, La Libertad department, Peru (7° 16' 28" S latitude; 79° 16' 49.3" W longitude, and 250 m.s.n.m. altitude). The ATCC 13311 strain of Salmonella enterica serovar Typhimurium was provided and verified using the VITEK 2 system in the laboratories of the Directorate of Institutes and Research Centers of the César Vallejo University. The taxonomic identification of the *Bidens pilosa* L. was carried out at the Herbarium Truxillense (HUT) of the National University of Trujillo (code Id. 60842).

A. Obtaining the ethanolic extract

The samples were transferred to the bacteriology laboratory of the Faculty of Microbiology and Parasitology (National University of Trujillo) to eliminate possible foreign compounds present in the plant material, then they were washed with distilled water and disinfected with 70% alcohol and sodium hypochlorite 3%; to finally eliminate excess hypochlorite through two new washes with sterile distilled water. The cleaned and disinfected leaves were allowed to dry for 72 hours at room temperature before being ground, pulverized, and sieved through a 0.5 mm mesh Amping Yuansheng brand. 200 g of *Bindes pilosa* L. leaf powder was used to macerate it in a dark bottle containing 1250 mL of 99.6% ethyl alcohol for seven days. During this period the bottle was kept in the dark and shaken for ten minutes two times per day, with an interval of 10 hours. The liquid phase was separated from the solid phase by vacuum filtration using Wattman N° 1 paper; the liquid part was concentrated in a rotary evaporator (120 rpm, 50 °C, and 1 atm pressure) and dried in an oven at 40 ° C. and dried in an oven at 40 ° C. The stock solution of 1000 mg/mL of the extract of *Bindes pilosa* L. was prepared using 4 g of dry extract and 4 mL of dimethyl sulfoxide (DMSO) (From this, the concentrations of 750, 500, 250, and 125 mg/mL were prepared). All different contractions were filtered using 0.20 µm Millipore membranes and finally stored in amber bottles at 4 ° C until further use.

B. Detection of Phytoconstituents

The metabolites were determined using the non-parametric crossover system: Presence: abundant (+++); moderate (++); low (+); and absence (-). The determinations of secondary metabolites and absorbance readings for biochemical quantifications were performed in triplicate.

To determine and quantify the phenolic constituents, the methodology described by Quiñones *et al*. (2015) and Valdivia et al. (2018) [25,26]. While for the determination of secondary metabolites, the methodology described by Valdivia et al. (2018) and Chigodi et al. (2013) [26,27] In determining the soluble phenolic content, 0.1 g of dry leaf powder and methanol were mixed (3 times up to a final volume of 1 mL). This mixture was shaken vigorously and centrifuged at 15,000 rpm for five minutes to remove the supernatant. The precipitate was homogenized with 1 mL of sodium hydroxide NaOH (2 mol/L) and then neutralized with 1 mL of hydrochloric acid HCl (2 mol/L). The concentration of soluble phenols was determined using chlorogenic acid (0.05 mol/L) as a standard, and the absorbance values were determined at 725 nm [25,26].

In the determination of the relative content of secondary metabolites to quantify Alkaloids, Steroids, Flavonoids, Saponins, and Terpenoids, the methodology described by Chigodi et al. (2013); Valdivia et al. (2018); Chafla-Moina & Silva-Déley (2023) and Song et al. (2022) [11,26-28]. In alkaloid determination, 5% hydrochloric acid is added in the extraction process and then precipitated using heavy metal salts such as those of Mayer's reagent (potassium iodide and mercury). The result obtained is the presence of a precipitate white to light yellow and is due to the presence of alkaloids. For the determination of steroids, 1 mL of the extract was mixed with 3 ml of chloroform ($CHCl_3$), and 2 mL of concentrated sulfuric acid (H_2SO_4) was carefully added to the sides of the test tube, where the formation of a red color on the upper layer and a green coloration in the H_2SO_4 layer indicated the presence of steroids in the extract. For the determination of flavonoids, 1 mL of sodium hydroxide (NaOH) 0.1 mol/L was added to 1 mL of the extract, and 1 mL of hydrochloric acid (HCl) 0.1 mol/L and the formation of a yellow color in the solution indicated the presence of flavonoids. In saponins determination, 1 mL of the extract was mixed with 3 ml of distilled water, and the mixture was heated to 100 ° C; foam formation and small bubbles showed the presence of saponins. For the determination of terpenoids, 1 mL of each extract was mixed with 1 mL of chloroform ($CHCl_3$) and 2 mL of concentrated sulfuric acid H_2SO_4; The presence of the red-brown coloration at the interface indicates the presence of terpenoids.

C. Antimicrobial activity

Activity antimicrobial was evaluated by the Kirby-Bauer method in wells [29]. The bacterial inoculum was obtained by a suspension of the colonies of *Salmonella typhimurium* ATTC 13311 (18-hour culture on Trypticase Soy Agar -TSA) in Physiological Saline Solution 0.85% (Sterile). A suspension of 0.50 was obtained in the DESICHEK plus equipment (equivalent to 1.5 x 10^8 cells/mL) [11,30]. With a sterile swab, the inoculum was spread on the surface of the Petri plate with Müller Hinton agar. Then wells were made in the agar (6 mm in diameter) where 50 µL of the extract was to be tested, in the same way and 50 µL of DMSO and 30 µg of amikacin were tested as negative and positive controls. All plates, were incubated at 37 ° C for 24 hours. After this time, the inhibition zones were measured and compared with data from the Clinical Laboratory Standard Institute (CLSI) [29]. The inhibition percentages were determined using the following equations:

$$\% \text{ inhibition} = \frac{A}{B}\, x\, 100 \qquad (1)$$

Where*:*
A: Diameter of the inhibition zone of the sample
B: Diameter of the control inhibition zone

In determining the Minimum Inhibitory Concentration (MIC), the macro dilution procedure in broth was used, observing the growth of the bacteria through the appearance of turbidity or color change of the culture broth [29]. The endpoint of the MIC was established by eye, observing the lack of turbidity in the broth, and comparing all tubes with the growth control. The macro dilution technique uses a working suspension of 1.5 x 10^6 cells/mL in Müeller Hinton (MH) broth and a series of 11 tubes with MH broth, where the final concentrations of the extract from tube N° 1 to 9 vary from 1000 to 3.9 mg/mL, due to the successive average dilutions carried out according to the procedure, tube No. 10 positive control (MH broth + bacterial suspension) and tube N° 11 negative control (contained MH broth).

In the determination of the Minimum Bactericidal Concentration (MBC), the MIC test tubes that did not show growth (turbidity) were used, and with the help of a sterile swab, an inoculum was taken and sown on the Müeller Hinton agar plates, and incubated at 37 °C for 24 hours. At the end of the incubation, bacterial growth was checked on the agar plates [29,31].

D. Statistic analysis

The data were statistically analyzed using SPPS v.26 software. This software sought to demonstrate whether the data obtained had a normal distribution using the Shapiro-Wilk normality test; if the groups evaluated are homogeneous using the homogeneity of variances test with the Levene statistic. If there is a difference between the means of the groups through an analysis of variance (ANOVA, $P<0.05$) and between which groups there is a significant difference using the statistical test such as Schcheffe's Post Hoc ($P<0.05$).

III. Results and Discussion

The use of medicinal plants as a treatment for infectious-contagious diseases in people in developing countries is an alternative to traditional medicine. The studies that seek to demonstrate the antimicrobial potential of *Bidens pilosa* L. on Gram-positive and Gram-negative bacteria seek to expand knowledge about which is the active compound responsible for

the antibacterial activity and add to the knowledge, which must constantly be updated according to the advances in modern medicine [10,12,28].

The phytoconstituents identified in the extract of *Bidens pilosa* L. are comparable with recent studies, where the presence of alkaloids, flavonoids, steroids, terpenes, saponins, and phenolic compounds [11,28,32]. These metabolites are considered the bioactive compounds that are present in the ethanolic extracts of a plant and are potentially responsible for the pharmacological and antimicrobial (antibacterial and antifungal) actions (see Table I), in our case a predominance of steroids and terpenoids was found. The presence of the five phytoconstituents present in the ethanolic extract of *Bidens pilosa* L. gives it its antibacterial activity because they can bind to the cell wall easily crossing the cell membrane, causing the denaturation of the protoplasmic proteins present in the bacteria; this is due to this the chemical structure of the compounds found: polyphenols, flavonoids, alkaloids, steroids, and terpenoids.

TABLE I
PHYTOCONSTITUENTS PRESENT IN THE ETHANOLIC EXTRACT OF *BIDENS PILOSA* L.

Phytoconstituents	Results *
Phenols	+
Alcaliodes	+
Steroids	++
Flavonoids	+
Saponins	–
Terpenoids	++

(*) Scale of Results: - Absent; + slightly present; ++ moderately present; and +++ very present.

Regarding the antibacterial activity of the ethanolic extract of *Bidens pilosa* L. against *Salmonella typhimurium*, it is observed that all concentrations showed inhibition on the growth of Salmonella typhimurium, the inhibition being greater in 3 of the 4 concentrations of the extract studied (Figura 1A), this is reflected when observing the inhibition diameters where the *Bidens pilosa* L. extract of 1000 mg/mL has a greater antibacterial activity with inhibition zones of 33.77 ±0.292 mm and a higher percentage of inhibition (130 %). Compared to the other concentrations of 500, 250, and 125 mg/mL. On the other hand, the 30 µg of amikacin (Ak) proved to be more effective (inhibition zones of 25.987 ±0.568 mm) than the concentration of *Bidens pilosa* L. of 125 mg/mL, with which inhibition zones of 10,097 ± 0.348 mm were obtained (see Table II). The ANOVA test results showed that the p-value is < 0.05; Therefore, the null hypothesis is rejected, and the alternative hypothesis that there is a significant difference between the experimental groups is accepted in Table II.

Table II
ANTIBACTERIAL ACTIVITY PRESENT IN THE ETHANOLIC EXTRACT OF *BIDENS PILOSA* L.

Groups (mg/mL)	n	% inhibition	[a]Med.	SD	V. min.	V. max.	[b]p-value
125	10	38.9	10.09	0.348	9.58	10.73	0.000
250	10	106.0	27.55	0.503	26.73	28.10	
500	10	111.7	29.03	0.366	28.42	29.61	
1000	10	130.0	33.77	0.292	33.18	34.14	
Ak (30 µg)	10	100.0	25.98	0.568	25.03	26.94	

[a]Medium value of the inhibition zone present in each concentration of the ethanolic extract of *Bidens pilosa* L.
[b]ANOVA test comparison ($p<0.05$) showed significant differences between the groups.

In comparison with a recent study. The results obtained were superior because in the study on the bactericidal action of the extract of *Bidens pilosa* L. against *S. typhimurium* carried out by Chafla-Moina & Silva-Déley (2023) reported inhibition zones of 8.3 ± 0.6 mm when they used an extract with a concentration of 10 mg/mL [10]. In comparison with similar research carried out with other microorganisms, the results obtained show that the ethanolic extract of the leaves of *Bidens pilosa* L. has an inhibitory effect because the inhibition zones exceeded those reported by Alonso (2019) and Pinilla et al. (2021), the latter in their study on the inhibitory effect of the ethanolic extract of *Bidens pilosa* L. against three bacteria, reported inhibition zones of between 4.08 - 11.63 mm for *S. aureus* (ATCC 25923); inhibition zones of between 2.25 and 7.29 mm for *L. monocytogenes* (ATCC 13932) and inhibition zones of between 6.17 and 13.04 mm for *B. cereus* ATCC 0299); also in all their experiments against concentrations between 100 to 1000 mg/mL [33,34].

In the present study, a strain of *Salmonella typhimurium* (Gram-negative bacteria) was used because previous studies have shown that Gram-negative bacteria have greater resistance to extracts than Gram-positive bacteria, as demonstrated by Panda et al. (2019) where Gram-positive pathogens were more susceptible to most extracts compared to Gram-negative species [35]. We must also mention that the diameters of the inhibition zones that are evaluated in different studies may differ due to the type of extract, amount of active ingredients and the environment where the plant develops, including abiotic factors such as climate, temperature, solar radiation and type of soil where the plant material for the studies grows and is obtained [12]. Another factor is the influence of the drying temperature on the extraction process. It has been reported that, in extraction processes where the drying temperature of the biological material was 50°C, better extraction yields were obtained for *Bidens pilosa* L [36].

To determine the degree of sensitivity of the different concentrations of the ethanolic extract of *Bidens pilosa* L. against *Salmonella typhimurium* ATCC 13311, the Duraffourd and Lampraz scale was used, which has been used to date in many research works; this scale it is possible to qualitatively determine the *in vitro* inhibitory effect of a substance based on the inhibition diameter [37]. The results of the average value of the inhibition zone (mm) present in Table II were used to

prepare the degree of sensitivity of *Salmonella typhimurium* ATCC 13311 to the different concentrations of the ethanolic extract of *Bidens pilosa* L. (see Table III). The results obtained show that the extracts of 1000, 500 and 250 mg/mL have a high sensitivity (+ + +) on *Salmonella typhimurium* by producing an inhibition zone that has a diameter > 20mm, while the 125 mg/mL extract has a low sensitivity (+) because it produces an inhibition zone of between 9-14 mm.

Table III
DEGREE OF SENSITIVITY OF *SALMONELLA TYPHIMURIUM* ATCC 13311 TO DIFFERENT CONCENTRATIONS OF THE ETHANOLIC EXTRACT OF *BIDENS PILOSA* L., ACCORDING TO THE SENSITIVITY SCALE OF DURAFFOURD ET AL.

Groups (mg/mL)	[a]Duraffourd and Lampraz Sensitivity Scale.			
	None (-)	Low Sensitivity (+)	Medium Sensitivity (++)	High Sensitivity (+++)
125	0 (0.0%)	10 (100.0%)	0 (0.0%)	0 (0.0%)
250	0 (0.0%)	0 (0.0%)	0 (0.0%)	10 (100.0%)
500	0 (0.0%)	0 (0.0%)	0 (0.0%)	10 (100.0%)
1000	0 (0.0%)	0 (0.0%)	0 (0.0%)	10 (100.0%)

[a]Duraffourd and Lampraz Scale: High Sensitivity (inhibition zone > 20 mm), Medium Sensitivity (inhibition zone 14-20 mm), Low Sensitivity (inhibition zone 9-14 mm), and None (inhibition zone < 8mm).

It is described that the minimum inhibitory concentration (MIC) is the lowest concentration of the extract which produces a 90 % reduction in colony growth. While the minimum bactericidal concentration (MBC) is the minimum concentration of the extract that produces at least a 99.9 % reduction in colony growth [38]. In the present study, the MIC of the extract was found to be 15.60 mg/mL and the MBC to be 31.25 mg/mL (Figura 1B). These results coincide with those obtained by Chafla-Moina & Silva-Déley (2023) who in their study on the bactericidal action of Bidens pilosa L. extract against *S. typhimurium* reported a MIC of 125 mg/mL and MBC of 250 mg/ mL. Concerning the solvent used to obtain the extract (99 % ethanol), it is not responsible for the antimicrobial activity, because the MIC and MBC of ethanol are negative, thus clarifying that the use of ethanol is as a solvent and not as an adjuvant.

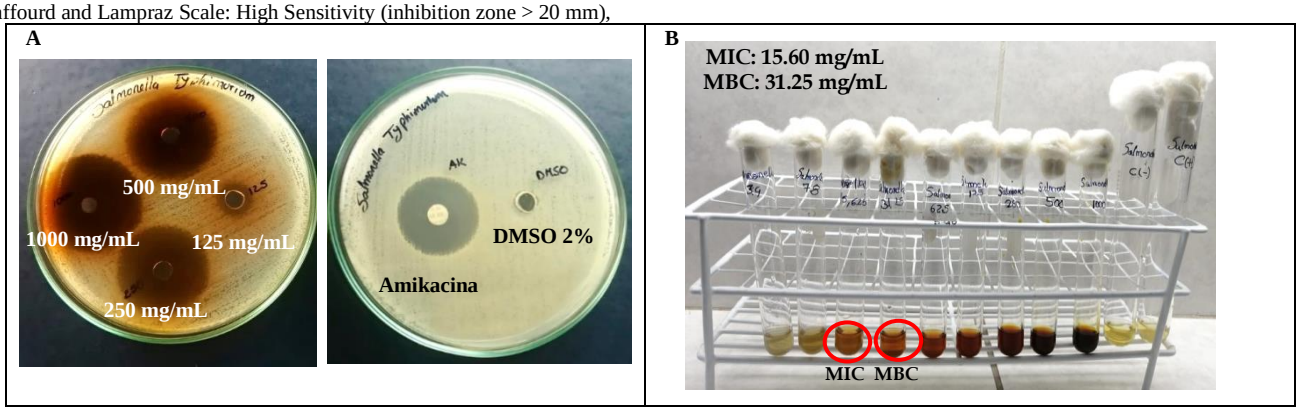

Fig. 1 Antimicrobial potential of the ethanolic extract of *Bidens pilosa* L. against *Salmonella typhimurium*. (A) Inhibition zones due to different concentrations of the ethanolic extract of *Bidens pilosa* L. on Petri dish inoculated with *Salmonella typhimurium* ATCC 13311; (B) Minimum inhibitory concentration MIC and minimum bactericidal concentration CMB of *Salmonella typhimurium* when challenged with the ethanolic extract of *Bidens pilosa* L.

Analysis statistical, it was found that the data obtained presented a normal distribution, divided into that in the Shapiro-Wilk normality test, the values obtained were higher than the value of p>0.05. The data obtained and evaluated by the group, with the Levene statistic, are presented homogeneously because the p-value obtained is >0.05 (p=0.204). It is observed that there is a significant difference between the experimental groups through the ANOVA test (p<0.005) and that there is a significant difference between groups because, in the statistical test such as Schcheffe's Post Hoc, the p values were < 0.05 in all the comparisons (see Table IV).

Table IV
TEST OF SIGNIFICANT DIFFERENCE BETWEEN TREATMENTS (SCCHEFFE TEST)

Comparison of treatments	**Mean differences**	**[a]p-value**
125 - 250 mg/mL	-17.458	0.000
125 - 500 mg/mL	-18.938	0.000
125 - 1000 mg/mL	-23.676	0.000
250 - 500 mg/mL	-1.480	0.000
250 - 1000 mg/mL	-6.218	0.000
500 – 1000 mg/mL	-4.738	0.000

[a]p-value < 0.05 showed significant difference between the groups

IV. CONCLUSION

The ethanolic extract of *Bidens pilosa L* has a high antimicrobial potential against *Salmonella typhimurium*, which causes gastrointestinal infections, and this antimicrobial activity is due to the presence of steroids and terpenoids.

The antibacterial activity was found in all concentrations of the extract, achieving inhibition zones from 10.097 mm ± 0.348 to 33.773 mm ±0.292, with inhibition percentages from 38.9 to 130.0%. In addition, a MIC of 15.6 mg/ml and a MBC of 31.25 mg/ml.

The results could be useful for the field of complementary or natural medicine, offering an option against bacterial resistance induced by the misuse of antibiotics.

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