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Chemical Composition and Antimicrobial Activity of Ethanolic Bark and Leave Extract of *Zanthoxylum Caribaeum* Lam. from Norte de Santander, Colombia

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Abstract: *Interest in the study of bioactive molecules present in plant organs such as root, leaves, stems or flowers, has increased significantly in search of applications in fields such as medicine and agriculture, mainly due to the bactericidal, antifungal and insecticides properties found in different plant species. In this study, leaf and bark extracts obtained at reduced pressure of Z. caribaeum were evaluated for their antibacterial and antifungal activity using the Kirby-Bauer agar disk diffusion technique. We found that extracts from the bark of this tree show a greater biological activity compared to extracts obtained from leaf, mainly against Grampositive bacteria such as S. aureus and S. mutans. However, there was no significant biological activity against gram-negative bacteria such as E. coli or Morganella sp. The composition of the extract determined by gas chromatography coupled to mass spectrophotometry (GC-MS), reveal the presence of the compound 3.5-dihydroxy-6-methyl-2.3-dihydro-4H-piran-one (32.8%) as a majority compound, which has been reported with antibacterial properties. The biological activity of the extracts of Z. caribeaum represents a potential source for the development of drugs for the control of microbial diseases.*

Keywords**:** *zanthoxylum caribaeum, antibacterial activity, Kirby-Bauer, ethanolic extracts*

1. Introduction

The need for new medicines that are effective and affordable to treat microbial diseases in developing countries is one of the health problems facing the world today [1], therefore, the development of new drugs is required to treat diseases caused by infectious bacteria [2]. Plants are the main sources of new medicines and can be an alternative to the usual medicines [3] However, of the 250,000 - 500,000 plant species, only a very low proportion has been studied for their pharmaceutical potential [4].

Plant-derived medicines remain an important resource, especially in developing countries where they are used to combat different types of diseases [5], particularly against pathogenic bacteria that are capable of obtaining antibiotic resistance factors, it is therefore necessary to seek and design alternative approaches for the control of pathogenic agents that may become resistant. One of these strategies is the search for bioactive phytochemicals with antibacterial activity [6]. Phytochemicals have been shown to be a good alternative to antibiotics and other chemicals due to serious side effects, the emergence of resistance and rare infections due to their overuse [7]. Research with plant extracts as medicines is of increasing interest due to the growing information on the antimicrobial activity of raw extracts, which could be better substitutes than conventional antibiotics [8].

Among the plant species as a promising source of plant extracts with antimicrobial activity is *Zanthoxylum caribaeum* Lam, popularly known in the department of Norte de Santander (Colombia) as "zorruno". Zanthoxylum species have shown biological activity against different pests or pathogens such as *Sitophilus zeamais* Mots, *Callosobruchus maculatus* [9], acaricidal activity [10, 11] and allelopathic properties [12]. Likewise, *Z. Monophyllum* showed biological activity against fungi that affect humans such as *Aspergillus terreus, A. flavus*, *Penicillium digitatum*, *P. funiculosum*, *P citinum, Paecilomyces* and *Candida albicans* [13].

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The present study using the Kirby-Bauer disk diffusion method, evaluated the antimicrobial potential of plant extracts of the species *Zanthoxylum caribaeum* Lam. against Gram-positive bacteria such as *Staphylococcus aureus*, an important human pathogen that causes a wide range of clinical infections [14], *Staphylococcus mutans*, an important etiological agent in dental caries and other pathologies [15], Gram-negative bacteria such as *Escherichia coli*, responsible for colibacillosis, a common disease of economic importance worldwide [16] and *Morganella Sp*., considered to be an unusual opportunistic pathogen that causes infections in post-operative wounds and the urinary tract [17]. The Kirby-Bauer disk diffusion method is a standard procedure for the susceptibility testing of bacterial isolates, it gives reliable results and can predict clinical efficacy of the antibiotics tested [18, 19]. Our results provide novel information on the active compounds present in *Zanthoxylum caribaeum* (Figure 1) in Norte de Santander (Colombia), as well as the antibacterial activity of its extracts.

Figure 1. Zorruno tree (*Zanthoxylum caribaeum* Lam) located in La Garita, Los Patios, Norte de Santander

2. Materials and methods

2.1. Ethanolic extract of *Z. caribeaum*

Z. caribaeum Lam samples with collection number COL:648, were collected at the La Garita location (7° 49’ 15” N, 72° 45’ 20.1” W) at 520 meters above sea level and an average temperature of 28°C. 500 g of plant material (leaves or bark) powder-free vegetable material was mixed in ethanol (Merck, Germany). Ethanol allows the identification of polar compounds. The mixture was left 48h in total darkness on a shaker (MAXQ 4450, Thermo Scientific™. Marieta, United States), 35°C and 100rpm. The extract was filtered under vacuum with filter paper (Qual. dia. 125mm, BOECO, Germany) using a vacuum pump (DOSIVAC, Buenos Aires, Argentina). The ethanolic extract was concentrated at reduced pressure using a rotary evaporator (IKA®RV10, Wilmington, United States) at 70rpm, 135mbar and 40°C. The concentrated extract was stored in amber bottles at 4°C for further analysis.

2.2. Gas Chromatography-Mass Spectrometry (GC-MS)

HPLC methanolic extract (80%) from the leaves and bark of *Z. caribaeum* was analyzed. The chromatographic analysis was performed on an AT 6890 Series Plus gas chromatograph (Agilent Technologies, MSD 5973, Santa Clara, United States), operated in full scan mode. The column used in the analysis was DB-5MS (5%-phenyl-poly (methylsiloxane), 60m x 0.23mm x 0.25µm). The injection was performed in Split mode (30:1) with the SPME device, using the Adams, Wiley and NIST databases.

2.3. Antimicrobial activity of *Z. caribaeum* Lam extracts

The Kirby-Bauer [20] method was used to evaluate the antimicrobial activity of ethanolic extracts of leaves and bark of *Z. caribaeum* against the pathogenic microorganisms *Staphylococcus aureus*,

Escherichia coli, *Streptococcus mutans* and *Morganella* sp. The antimicrobial activity of the ethanolic extracts of leaves and bark of *Z. caribaeum* was quantitatively evaluated for the presence of halos of inhibition and was statistically analyzed. Concentrated extract of *Z. caribaeum* Lam were used to prepare dilutions at 1:1, 1:2, 1:3, and 1:4 (extract:ethanol). Sensidiscs were impregnated with dilutions and the concentrated extract for the evaluation of their antibacterial activity against Gram-positive bacteria (*S. aureus, S. mutans*) and Gram-negative bacteria (*E. coli, Morganella sp*). Each trial consisted of 6 repetitions for statistical validation. A concentration of 1mg/mL of Kanamycin was used as a positive control and water and alcohol were used as negative controls. Plates with bacteria and treatments were incubated for 18-24 h at 37°C, and then inhibition halos were measured. Results are expressed as sensitive (S), intermediate or moderately sensitive (I), and resistant (R)) (Pascual et al., 2001). The treatments used were: T1: Positive control; T2: Ethanol; T3: Water; T4: Concentrated extract; T5: 1:1 extract ethanol; T6: 1:2 extract ethanol; T7: 1:3 extract ethanol; T8: 1:4 extract ethanol

3. Results and discussions

3.1. Major compounds of the leaf and bark extracts of *Zanthoxylum caribaeum*

In the leaf extract of *Z. caribeaum* from Norte de Santander, α-trans-farnesene was identified as the majority compound with an abundance percentage of 33.5%., followed by Trans-β-karyophyllene with 28.1%. Table 1 shows a comparison of the compounds found in this study and those found by Nogueira et al., in 2014 (Table 1). Figure 2 shows the Chromatogram of ethanolic extract of z. caribeaum leaves.

Table 1. Comparison of the major compounds obtained from Zanthoxulum caribaeum methanolic extract by GC-MS with those obtained in essential oil [21]

T_R (Min)	Compound	Relative amount This study %	Relative amount Nogueira *et al.* %
36.91	*trans-β-Caryophyllen*	28.1	4.3
38.13	α-Humulene	3.0	1.1%
38.64	γ-Muurolene	8.1	0.9
39.23	Valencene	3.1	NR
39.32	α-*trans*-Farnesene	33.5	NR
39.89	γ-Cadinene	2.8	NR
39.98	S-Cadinene	9.7	NR
40.11	Calamenene	1.2	NR
40.43	NI.	2.1	-
42.07	Caryophyllene oxide	8.3	NR

Several of the compounds found in *Z. Caribeaum* described in Table 1 have been described with different biological properties. Among the majority. α-trans-farnesene has been attributed as an antibacterial agent against microorganisms such as *Bacillus cereus*. *Pseudomonas aureofaciens*. *Aspergillus ochraceus*. *Candida pseudotropicalis*. *Kluyveromyces lactis* and *Fusarium moniliforme* [22].

On the other hand. the results obtained from the chromato3.5-Dihydroxy Methyl-6-methyl-2.3-hydroxy-4H-piran-one graphic analysis of the bark extract of Z. caribaeum Lam. showed as the majority compound 3.5-Dihydroxymethyl-6-methyl-2.3-dihydroxy-4H-piran-one with a 32.8% abundance and a retention time of 23.88 min followed by the hydroxymethyl-furfurfurfural with 19.2% and a retention time of 26.96. and and 2-undecanone with 1.9% abundance (Table 2). Figure 3 shows the Chromatogram of ethanolic extract of *Z. caribeaum* bark.

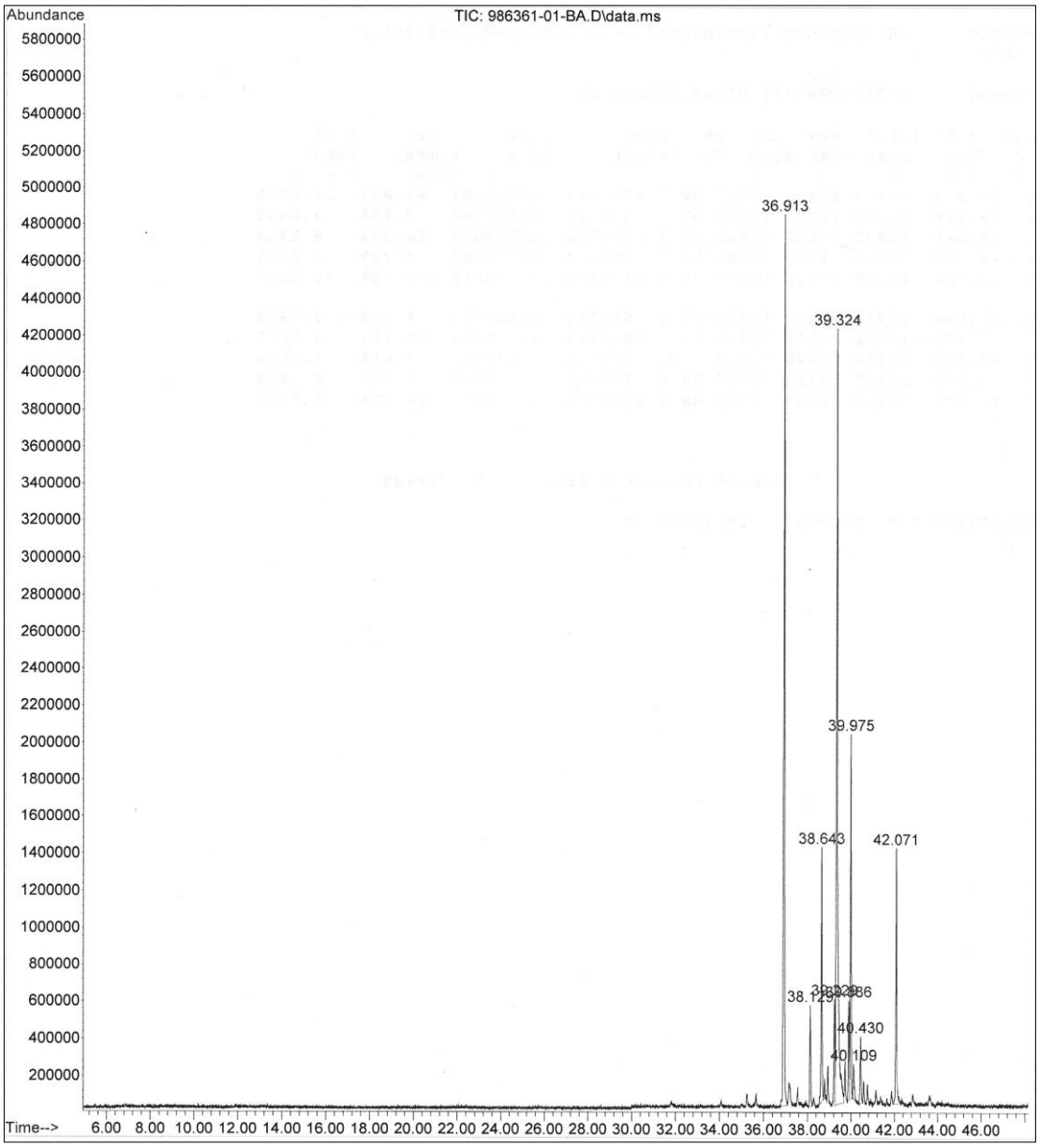

Figure 2. Chromatogram of the ethanolic extract of *Z. Caribeaum* leaves showing the peaks corresponding to the identified major metabolites

Table 2. Presumptive identification by GC-MS. retention times (tR). and relative amount (%) of components present in the bark extract of Z. caribaeum

tR / min	Presumptive identification	Relative amount %
23.88	3.5-Dihidroxi-6-metil-2.3-dihidro-4H-piran-4-ona	32.8
26.96	Hidroximetil-furfural	19.2
29.17	Undecanona	1.9

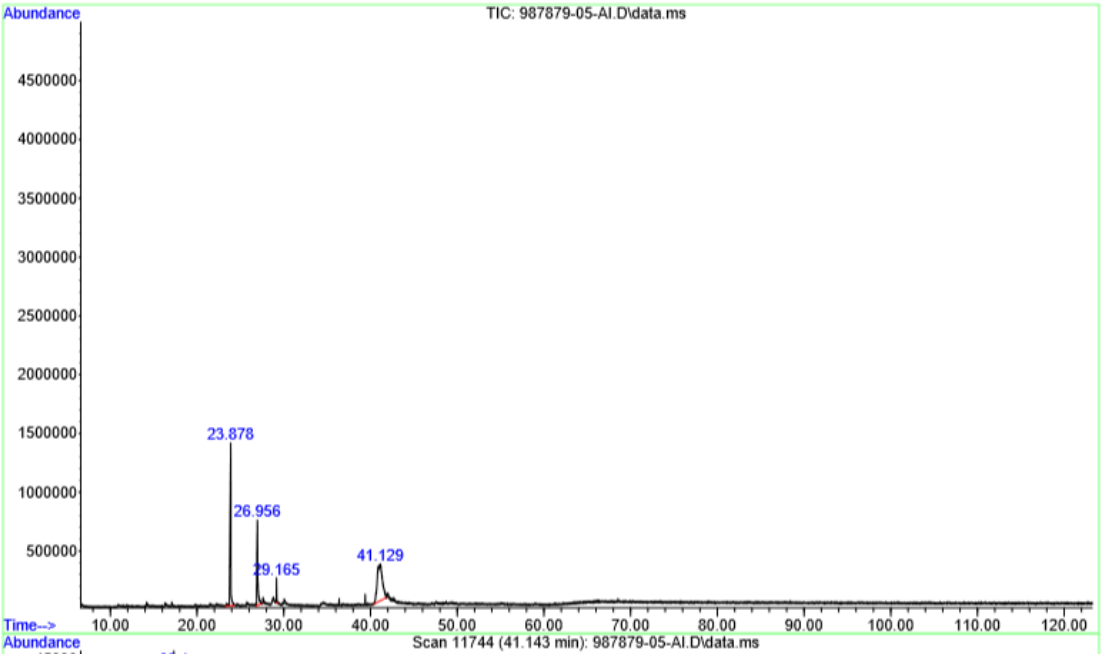

Figure 3. Chromatogram of the ethanolic extract of *Z. caribeaum* bark showing the peaks corresponding to the identified major metabolites

It was possible to corroborate that the majority compound present in the bark extract of *Z. caribaeum* belongs to the family of coumarins. performing a specific test for these secondary metabolites. This test consisted of adding diluted KOH to the concentrated extract. the reaction was considered positive since it presented blue fluorescence at a wavelength of 365nm [23, 24] (Figure 4).

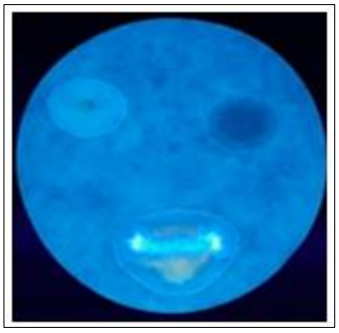

Figure 4. Test for coumarins**.** Blue fluorescence at 365 nm. Considered positive for the presence of coumarins

3.2. Effect of ethanolic extracts of *Z. caribaeum* against *Staphylococcus aureus*

The results of inhibition against S. aureus bacteria indicate different effects when comparing the effect of leaf extract and bark extract. The concentrated leaf extract (T4) caused a growth inhibition of the bacteria with a halo greater than 5 mm (Figure 5).

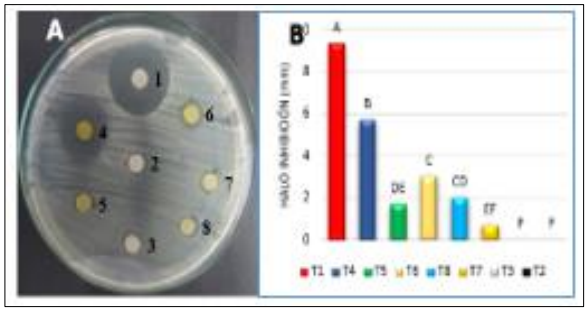

Figure 5. Antibacterial activity of dilutions of *Z. caribaeum* leaf extract in ethanol (extract:ethanol) against *S. aureus*. A. Bioassay: T1. Positive control. T2 Ethanol T3 H_2O. T4 concentrated extract. T5. Dilution 1:1. T6. Dilution 1:2. T7. Dilution 1:3. T8. Dilution 1:4. B. The treatments present significant differences $P < 0.05$ with the Tukey test. n=6. The bars represent the confidence intervals for $\alpha = 0.05$

The antibacterial activity of *Z. caribaeum* bark extract is higher. as growth inhibition was present in all treatments. However. treatments four (concentrated extract. T4) and five (Dilution 1:1. T5) induced the greatest halos of inhibition with 8mm and 6mm respectively. Treatments. T6. Dilution 1:2. and T7. Dilution 1:3. induced inhibitions equal to or less than 2mm. (Figure 6).

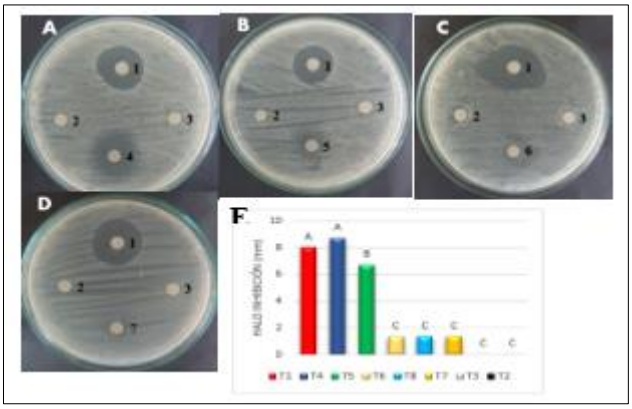

Figure 6. Antibacterial activity of dilutions of *Z. caribaeum* bark extract in ethanol (extract:ethanol) against *S. aureus*. A. Bioassay: T1. Positive control. T2 Ethanol T3 H_2O. T4 concentrated extract. T5. Dilution 1:1. T6. Dilution 1:2. T7. Dilution 1:3. T8. Dilution 1:4. B. The treatments present significant differences $P < 0.05$ with the Tukey test. n=6. The bars represent the confidence intervals for $\alpha = 0.05$

Treatments with bark extract presented better results against Grampositive bacteria *S. aureus*. which was sensitive and moderately sensitive for concentrated extract (T4) and 1:1 dilution (T5) treatments. respectively.

3.3. Effect of ethanolic extracts of *Z. caribaeum* against *Streptococcus mutans*

S. mutans showed resistance to ethanolic extracts of leaves of *Z. caribaeum*. as no inhibition could be seen in any of the treatments performed (Figure 7).

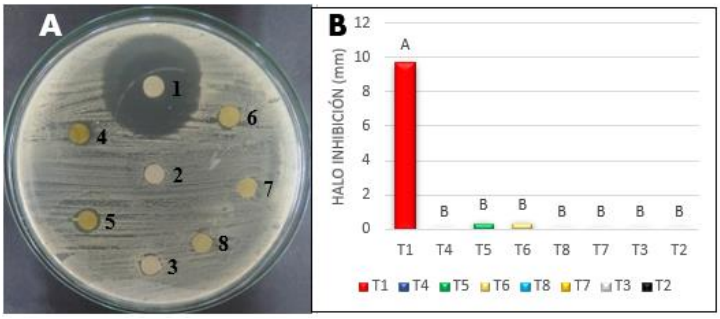

Figure 7. Antibacterial activity of dilutions of *Z. caribaeum* leaf extract in ethanol (extract:ethanol) against *S. mutans*. A. Bioassay: T1. Positive control. T2 Ethanol T3 H_2O. T4 concentrated extract. T5. Dilution 1:1. T6. Dilution 1:2. T7. Dilution 1:3. T8. Dilution 1:4. B. The treatments present significant differences $P < 0.05$ with the Tukey test. n=6. The bars represent the confidence intervals for $\alpha = 0.05$

However. as was the case with *S. aureus. Z. caribaeum* bark extract was found to inhibit the growth of *S. mutans* with treatments four (concentrated extract. T4) and five (Dilution 1:1. T5) (Figure 8).

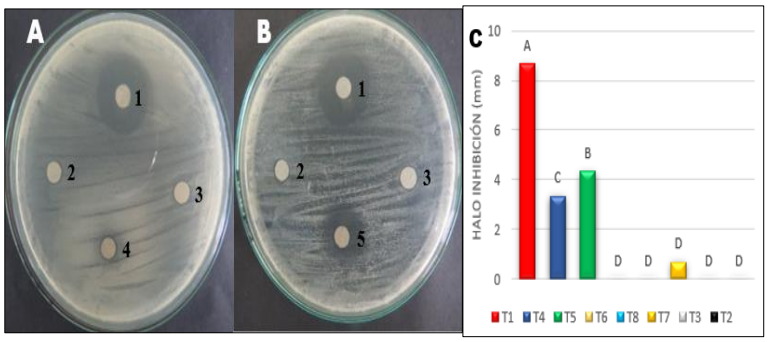

Figure 8. Antibacterial activity of dilutions of *Z. caribaeum* bark extract in ethanol (extract:ethanol) against *S. mutans*. A. Bioassay: T1. Positive control. T2 Ethanol T3 H_2O. T4 concentrated extract. T5. Dilution 1:1. T6. Dilution 1:2. T7. Dilution 1:3. T8. Dilution 1:4. B. The treatments present significant differences $P < 0.05$ with the Tukey test. n=6. The bars represent the confidence intervals for $\alpha = 0.05$.

3.4. Effect of ethanolic extracts of *Z. caribaeum* against *Escherichia coli*

Inhibition bioassays performed with ethanolic leaf and bark extracts of *Z. caribeaum* did not yield positive results against the Gram-negative bacterium *E. coli* (Figure 9).

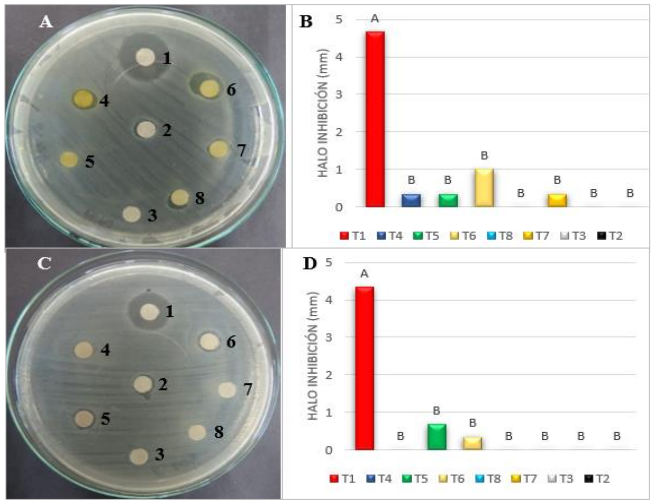

Figure 9. Antibacterial activity of dilutions of *Z. caribaeum* leaf and bark extract in ethanol (extract:ethanol) against *E. coli*. A (Leaves) and C (bark). T1. Positive control. T2. Ethanol. T3. water. T4. Concentrated extract. T5. Dilution 1:1. T6. Dilution 1:2. T7. Dilution 1:3. T8. Dilution 1:4 B and D. Tukey multiple comparison analysis for leaves and bark respectively. The data show significant differences $P < 0.05$ with the Tukey test n = 6. The bars represent the confidence intervals for $\alpha = 0.05$

3.5. Effect of ethanolic extracts of *Z. caribaeum* against *Morganella sp.*

As with the Gram-negative bacterium *E. coli*. bioassays performed with the leaf and bark ethanolic extracts of *Z. caribeaum* were not evidenced inhibition of the *Morganella sp* bacteria (Figure 10).

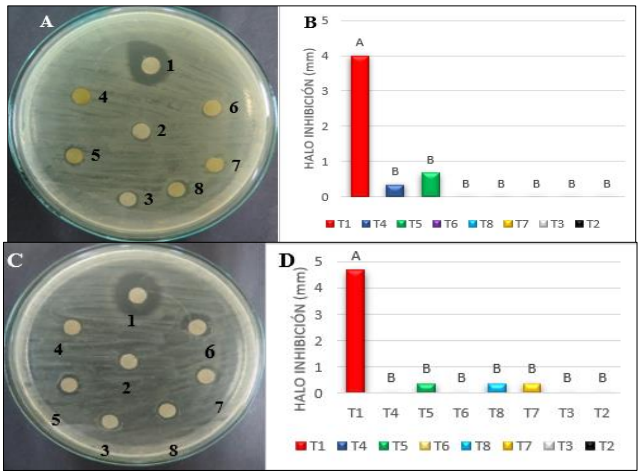

Figure 10. Antibacterial activity of dilutions of *Z. caribaeum* leaf and bark extract in ethanol (extract:ethanol) against *Morganella Sp.* A (Leaves) and C (bark). T1. Positive control. T2. Ethanol. T3. water. T4. Concentrated extract. T5. Dilution 1:1. T6. Dilution 1:2. T7. Dilution 1:3. T8. Dilution 1:4 B and D. Tukey multiple comparison analysis for leaves and bark respectively. The data show significant differences $P < 0.05$ with the Tukey test $n = 6$. The bars represent the confidence intervals for $\alpha = 0.05$

The antibiogram method with dilutions of the concentrated extract of the plant *Z. Caribeaum*. from the department of Norte de Santander. Colombia. using the Kirby-Bauer technique showed that Gram-positive bacteria such as *S. aureus* and *S. mutans* have a greater sensitivity compared to Gram-negative *E. coli* or *Morganella sp*. bacteria. The bark extract evidenced a greater biological activity against sensitive bacteria. compared to leaf extract. The sensitivity in *S. aeurus* and *S. mutants* can be attributed to the fact that Gram-positive bacteria are monoderms surrounded by a cytoplasmic lipid membrane and lack the presence of an outer cell membrane that is present only in Gram-negative bacteria [25]. This may be due to what Gupta suggested [26] who indicates that antibiotic selection pressure was an important selective force in prokaryotic evolution and likely played a central role in the evolution of Gram-negative didermo bacteria. Thus. the outer membrane of Gram-negative bacteria plays an important role as a protective mechanism against antimicrobial agents providing an extra layer of protection [27].

Through mass-coupled gas chromatography studies it was evident that the ethanolic extracts of leaf and bark of *Z. Caribeaum* present differences in their chemical composition and that the bark extracts presented greater biological activity against sensitive bacteria. The majority compound in bark extracts is 3.5-Dihydroxy-6-methyl-2.3-dihydro-4H-pyran-4-one with 32.8% followed by Hydroxymethyl-furfural with 19.2% and undecanone with 1.9% because it has a pyran 3.5-Dihydroxy-6-methyl-2.3-dihydroxy-4H-pyran-4-one is a secondary metabolite belonging to complex coumarins. Coumarins exhibit antibacterial activity [28-33]. So the majority compound present in the bark extract of *Z. Caribeaum* could be responsible for the antimicrobial activity observed against Gram-positive bacteria.

4. Conclusions

The ethanolic extract of *Z. caribaeum* bark has good antibacterial activity in vitro against Gram-positive bacteria *Staphylococcus aureus* and *Streptococcus mutans*. However the Gram-negative bacteria (*Escherichia coli* and *Morganella* sp.) presented resistance to all treatments of leaf and bark extracts. The results are promising to use *Z. Caribeaum* as a source for the production of new antibacterial products against Gram-positive bacterias.

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