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Antimicrobial potential of leaf extracts of Cariniana legalis

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Short Report

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Title: Antimicrobial potential od leaf extracts of *Cariniana legalis*

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Abstract

The study investigated the antimicrobial potential of leaf extracts of *Cariniana legalis* (Pink Jequitibá), a plant of the Lecythidaceae family, aiming at the discovery of new compounds with antibacterial activity. The leaves were subjected to the extraction process using different polar solvents, with the hydroalcoholic extract showing the greatest antimicrobial activity. The extract was fractionated by liquid-liquid separation and silica chromatography, and the fractions were evaluated for their antimicrobial capacity by inhibition halo in Escherichia coli, Staphylococcus aureus and Ralstonia solanacearum. The liquid-liquid separation fraction obtained with ethyl acetate showed the best result and was subjected to chromatographic separation. The obtained fractions were standardized, and the results of this step indicated potential to inhibit bacterial growth and biofilm formation. The GC/MS technique revealed compounds with distinct properties that are antimicrobial candidates, possessing both ester and amide organic functions in their structures. This work led to a study demonstrating the antimicrobial capacity of *Cariniana legalis* leaf extracts.

Introduction

Bacteria are susceptible to mutations, which make them resistant to previously known drugs, such as strains with a high rate of danger, such as methicillin-resistant Staphylococcus aureus (MRSA) (RODRIGUES et al., 2024) and Klebsiella pneumoniae carbapenemase (KPC) (LIU et al., 2024), which are resistant to the most modern antibiotics. In addition to the need to obtain antimicrobials capable of eliminating these bacteria, it is essential that they present a low level of toxicity to the user so that they can be a potential antibiotic.

Not only human health, but also the agricultural sector needs a more natural approach against pathogens, since the indiscriminate use of antimicrobial agents can result in new multi-resistant bacteria and compromise an entire production chain. Ralstonia solanacearum, also known as bacterial wilt, is one of the most severe diseases affecting tomato crops in several regions of the world. This bacterial species has a wide range of hosts within the Solanaceae family, which significantly increases its potential for economic and ecological damage, although integrated management and genetic resistance are important as effective strategies to mitigate the impacts of this devastating disease on tomato crops, thus seeking to ensure sustainability and food security in these critical agricultural areas (TAHAT; SIJAM, 2010).

The genus Cariniana sp. (Lecythidaceae), in the biochemical context, has great potential due to the richness of its secondary compounds. Among the main ones are flavonoids, triterpenes and phenolic compounds, known for their antioxidant and anti-inflammatory activities. The antimicrobial activity also stands out, suggesting its use in phytotherapy and in the development of new therapeutic agents (JANOVIK et al., 2012). The combination of these properties makes the plant a promising source of bioactive substances that can be applied in the treatment of inflammatory diseases, bacterial infections and even in combating oxidative stress, with a positive impact on human health (FERREIRA et al., 2021). Therefore, the present study aimed to explore the antimicrobial effect in polar extracts of *Cariniana legalis*, known as Jequitibá-rosa.

Materials and Methods

Registration of material in a herbarium

A specimen of the plant used in this study was registered in the Herbarium of the Federal University of Viçosa, as stated in the records: VIC 58146

Preparation of extracts

The experiment was divided into two stages. In the first, intact plant leaves, without lesions, were selected and dried in a ventilated oven at 50°C for 96 hours, until they became brittle. After drying, the material was crushed to obtain a fine powder, aiming to optimize the extraction process. In the second stage, the crushed material was subjected to a ratio of 1.0 g of sample to 10 mL of solvent, with constant agitation at 160 RPM for 1 hour. The resulting mixture was filtered through gauze and filter paper, and the filtrate was dried in an oven at 50°C to remove the solvent. The mass of the dry extract was determined and stock solutions were prepared and centrifuged at 10,000 G for 10 minutes. The supernatant was filtered with a 0.22 µm membrane and stored in microtubes at -20°C for future use.

Liquid-liquid separation, thin layer chromatography (TLC) and fractionation by open column chromatography.

The purification of the extracts was carried out using two specific methods: the first by a liquid-liquid separation step and the second by silica gel chromatography (BIBI et al., 2016). For the liquid-liquid fractionation process, a 50 ml sample of the hydroalcoholic extract was prepared and completely dried at 60°C, the mass obtained was resuspended in 25 ml of distilled water.

The sample underwent liquid-liquid purification, which was conducted by increasing the polarity of the separation phases, as shown in figure 1. After the separation with all solvents, the phases were dried and their mass measured for standardization, and the antimicrobial activities were tested by inhibition halo. After the antimicrobial evaluation, the sample obtained in the liquid-liquid separation was evaluated on a silica gel TLC on a polyester support, with the aim of selecting an ideal solvent to separate components of the sample in question.

A preliminary evaluation in TLC (thin layer chromatography) of the fraction obtained in ethyl acetate indicated the use of ethanol as the mobile phase and a solution of water with acetonitrile (1:1) as the total eluent of the sample. Thus, the column was packed and conditioned with ethanol to begin the procedure in two stages: a separation stage with ethanol that continued for 60 minutes and then its solvent was changed to water:acetonitrile (1:1), starting the second stage for complete elution of the sample.

Chromatography was performed using Silica Gel S (Kieselgel S), by packing a 50 cm3 volume (1.0 cm radius, 15.923 cm height) with HPLC grade solvent. After packing, the column was eluted 3 times its volume (150 ml) with the solvent for conditioning, and then the sample was applied for elution at a flow rate of 1.0 mL/min. The collected tubes were dried in an oven at 40ºC for 48 hours and their contents were resuspended with distilled water to evaluate the antimicrobial activity by growth curve.

During both stages, the flow rate was maintained at 1.0 ml/minute, totaling 60 samples in each stage (total of 120 fractions). The sample applied was 2.0 ml at a concentration of 30 mg/ml. The fractions from column chromatography, after being dried, were resuspended at a concentration of 0.07 mg/mL, followed by antimicrobial activity tests using a growth curve, whose final concentration in the antimicrobial test was 0.0175 mg/mL.

Antimicrobial activity by inhibition zone of liquid-liquid separation

The disk diffusion experiment was carried out in accordance with the standards of the European Committee on Antimicrobial Susceptibility Testing (https://www.eucast.org). To perform this antimicrobial activity screening, the Muller-Hinton agar medium was deposited in 92X16mm petri dishes with 4mm. Using a swab, the inocula previously prepared on the McFarland scale with absorbance of 0.5 at 600 nm were transferred to the plate. The plates were left ajar for drying and adhesion of the inoculum to the culture medium for five minutes, and the sterile filter paper discs of 6.0 mm in diameter were placed equidistantly, followed by the fractionated extract, which was applied in 10.0 µL of each fraction and the plates incubated for 48 hours at 37ºC.

Analysis of the fractions by Gas Chromatography coupled to Mass Spectrometry (GC/MS)

The analyses were performed in a gas chromatograph (Shimadzu) equipped with a quadrupole mass spectrometer (MS) detector (GCMS-QP2020), an injection system (AOC-20i.), Shimadzu® brand. The chromatographic separation of the analytes was performed in a capillary column (SH-Rtx5MS), whose stationary phase is composed of 5% phenyl and 95% dimethylpolysiloxane, 30 m long, 0.25 mm internal diameter and 0.25 mm film thickness. Helium gas (99.999% purity, White Martins) was used as a carrier at a flow rate of 1.08 mL/min.

The column temperature programming consisted of maintaining the initial temperature at 40°C for 2 minutes, followed by 150°C for 1 minute and 280°C for 5 minutes, and ending with 320°C for 3 minutes. The total analysis time was 40 min. The injector temperature was maintained at 300°C. A 1 µL sample volume was injected into the chromatograph using the 50% split injection mode. The mass spectrometer (MS) was operated in positive ionization (PCI) mode, electron impact 70 eV. A cutoff time of 3.0 min was set to avoid instrument damage. The detector interface temperatures were set at 340 °C, and the ionization source at 230 °C.

Bacterial growth curve in the presence of the obtained fractions

The experiment followed the growth curve standards, whose initial inoculum is 106 colony forming units (CFU): the negative control (150 µL of MH broth, 50 µL of bacterial inoculum), positive control (100 µL of MH broth, 50 µL of chloramphenicol diluted in MH broth at 2.0 mg/ml, 50 µL of bacterial inoculum) and the assays to test the chromatography fractions (100 µL of MH broth, 50 µL of the fraction diluted in MH broth, 50 µL of bacterial inoculum). The plate reading was measured on a Thermo Multiskan GO spectrophotometer (1510-01215) at a wavelength of 600 nm and, when necessary, subjected to the 0.01% resazurin test to assess cell viability (TWIGG, 1945).

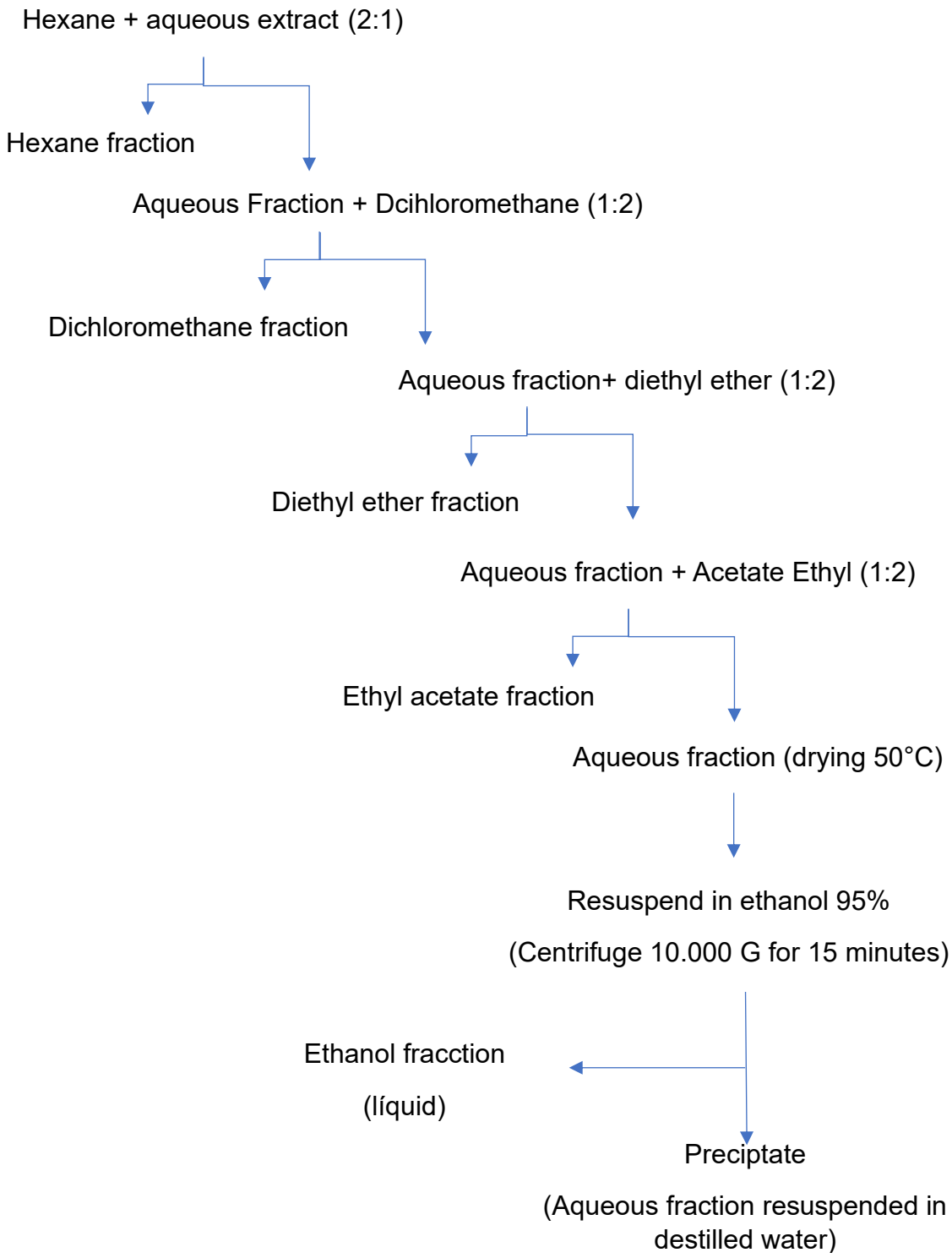


Figure 1: Flowchart of the liquid-liquid purification steps of crude extract of *Cariniana legalis* leaves

Bactericidal activity (MBC) of liquid chromatography fractions

The MBC was measured quantitatively using the serial dilution technique to count colony-forming units per milliliter (CFU/ml). The bacteria that were subjected to the growth curve with fraction treatment had their colony count determined by the serial dilution method of each assay for a more robust CFU count. Dilutions from 10-1 to 10-9 were prepared in 0.85% saline solution (NaCl), and a 50 µL fraction of each dilution was applied to plates containing Muller Hinton agar, which were subjected to the spread plate method and incubated at 37ºC for 48 hours. The number of colonies was measured by counting on a Petri dish, which ranged from 25 to 300.

Evaluation of the effect of fractions on biofilm formation.

The method of Antunes et al (2010) adapted by Trentin (2011) was adopted, which involves the use of crystal violet in sterile 96-well microtiter plates. In each well, 40 microliters of the bacterial suspension, whose absorbance at 600 nm was 0.5 on the McFarland scale, 20 µl of the prepared fraction, and 140 µl of NYD broth were added. After incubation at 37 °C for 24 hours, the contents of the wells were discarded and the wells were washed three times with sterile PBS (phosphate buffer) saline solution at pH 7.2. The adhered bacteria were then fixed by heating at 60 °C for 1 hour. The biofilm formed was stained with a 0.4% (m/v) crystal violet solution for 15 minutes at room temperature. The crystal violet pigment bound to the biofilm was extracted with 99.5% ethanol, and after 30 minutes, the absorbance was evaluated at 570 nm using a plate reader. The control was considered as 100% of biofilm formation, and instead of the fraction, 20 µl of water were added to the wells. Values above 100% indicated greater biofilm formation compared to the control.

Statistical analysis

The results were compared with each other and with the control using the Tukey test, with a significant value of $p \leq 0.05$.

Results and discussions

Extract selection, purification and fractionation in chromatography

The evaluation of the inhibition zone first followed a screening with three organisms: Escherichia coli (ATC 35218), Staphylococcus aureus (ATC29213) and Ralstonia solanacearum (LPF0710). figure 2 shows that the ethanolic extract stood out with superior antimicrobial activity to the others. Through screening, we selected the ethanolic extract for this study and its liquid-liquid fractionation followed with a concentrated sample, so that the antimicrobial compounds, after chromatography, were in quantities sufficiently sensitive for the selected bacteria. After drying, the masses obtained were standardized (Table 1) and the inhibition halo tests were performed, as shown in figure 3, verifying that the fraction obtained in ethyl acetate contained the antimicrobial compounds.



Figure 2: evaluation of the antimicrobial activity of pink jequitibá leaf extracts. 1 - ethanolic extract / 2 - methanolic extract / 3 - aqueous extract / A - water / N - negative control

Table 1: Mass of liquid-liquid separation fractions of the ethanolic extract of *Cariniana legalis* leaves

Organic Solvent	Mass obtained (mg)	Standardized Concentration (mg/mL)
Hexane	60,0	30,0
Dichloro methane	15,0	10,0
Diethyl ether	19,0	10,0
Ethyl acetate	60,0	30,0
Ethanol	544,0	100,0
Water	252,0	100,0

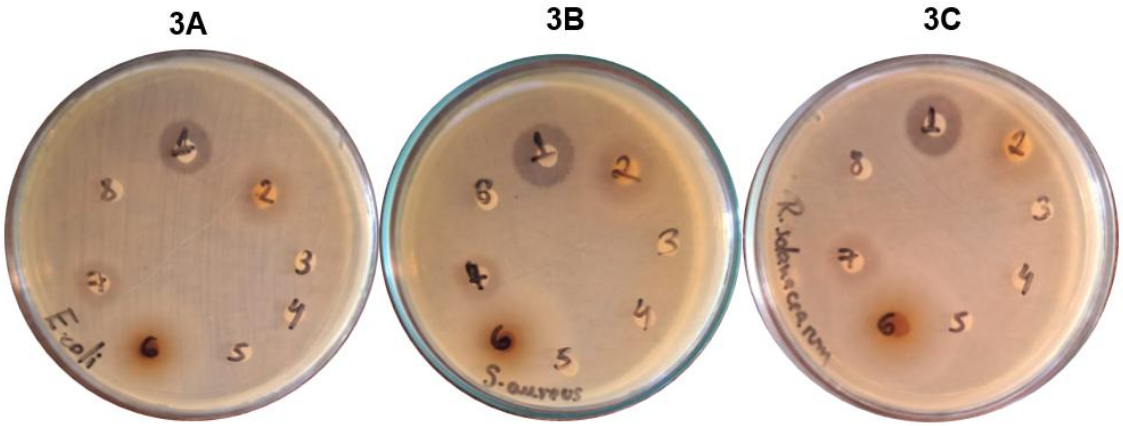


Figure 3: Screening of antimicrobial activity by inhibition zone of liquid-liquid separation fractions of the ethanol extract of C. legalis leaves in E. coli (A), S. aureus (B), R. solanacearum (C) Legend: 1: positive control (chloramphenicol 2.0 mg/ml) / 2: crude ethanol extract / 3: hexane fraction / 4: dichloromethane fraction / 5: diethyl ether fraction / 6: ethanol fraction / 7: ethyl acetate fraction / 8: aqueous fraction.

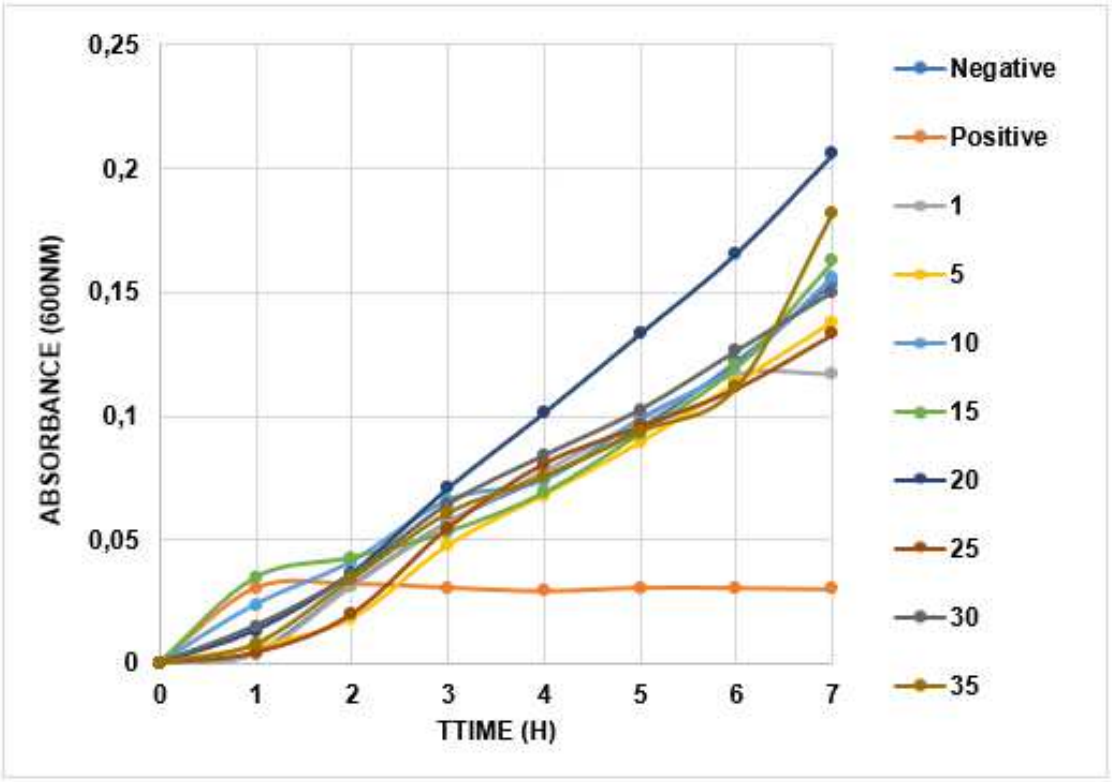
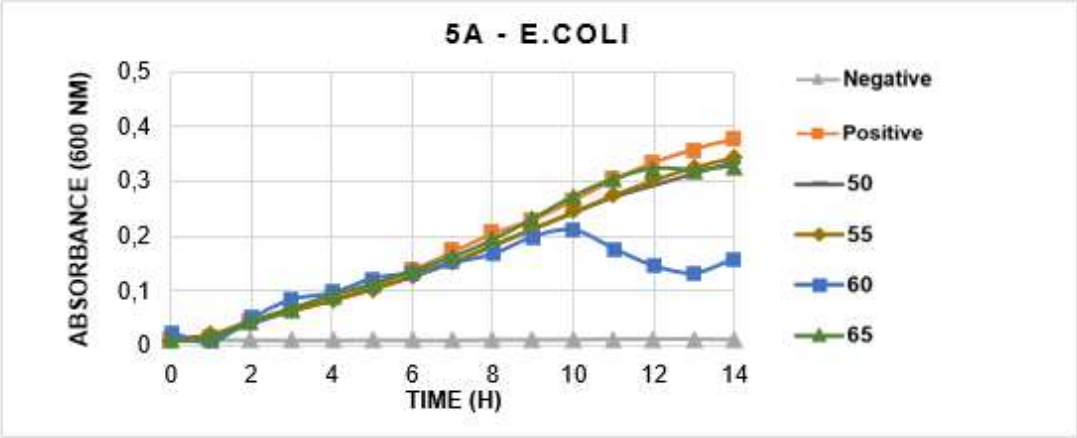
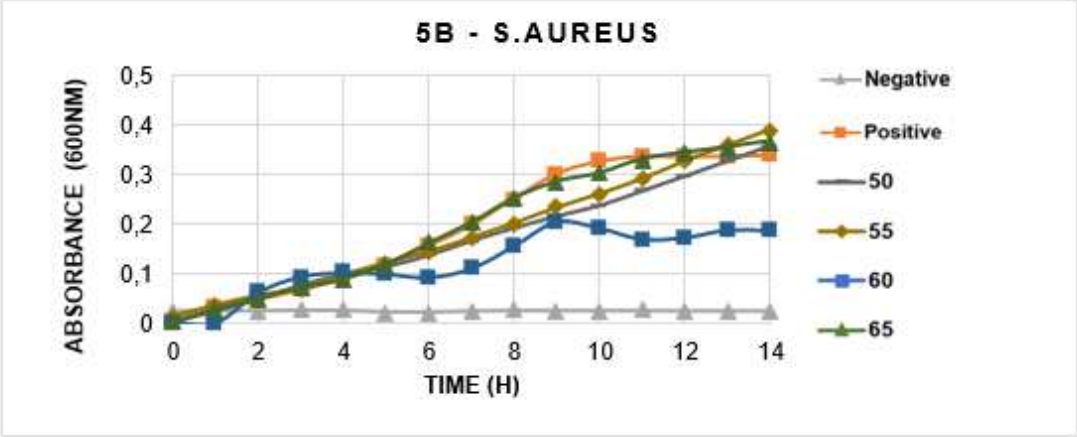


Figure 4: Bacterial growth of E. coli subjected to treatment with chromatographic fractions of the ethanolic extract of C. legalis leaves. Negative Control: chloramphenicol 2.0 mg/ml / Positive control: bacterial growth without treatment / Numbers: chromatographic fractions of the hydroalcoholic extract of pink Jequitibá.

Fraction number 60 stood out as the most promising growth inhibitor, as observed in figure 4. To verify that nearby fractions could also contain antimicrobial compounds, a new bacterial growth test was performed with fractions 50, 55, 60 and 65 for confirmation, with all bacteria, as shown in figure 5. The bacterial growth observed in figure 5 indicates that not all chromatography fractions inhibited bacterial growth, as is the case with samples 50 and 55, but fraction 60 still stood out for all bacteria and fraction 65 for R. solanacearum.





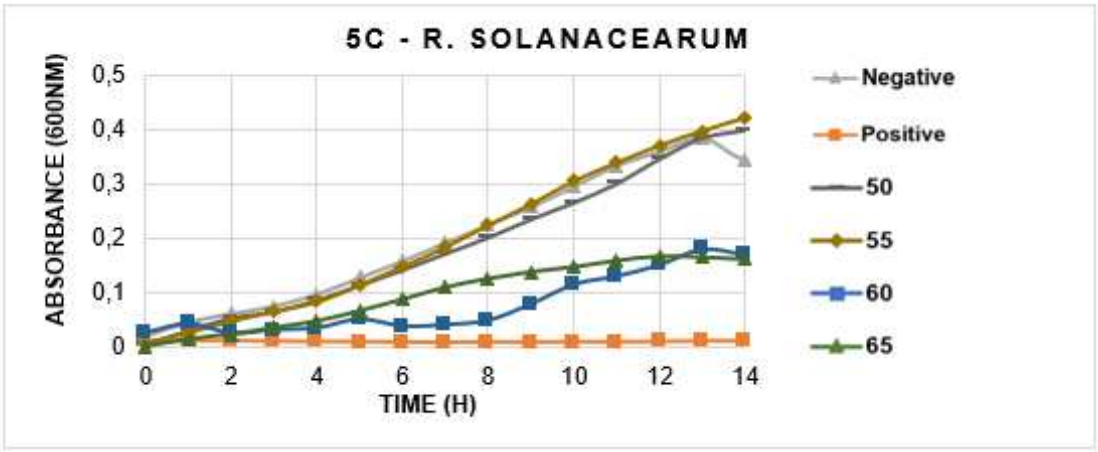


Figure 5: Bacterial growth of E. coli (A), S. aureus (B) and R. solanacearum (C) subjected to fractions 50, 55, 60, 65 of chromatography of the hydroalcoholic extract of C. legalis leaves. Negative control: chloramphenicol 2.0 mg/ml / Positive control: bacterial growth without treatment / Numbers: chromatography fractions of the hydroalcoholic extract of pink Jequitibá

The growth curve was redone with samples 50 to 65, with a variation of 1 to 1 (figure 6), and only those that stood out below the negative control were selected for plotting on the graph and counting the colony-forming units, in order to verify the real effect of the growth reduction in the samples in a quantitative manner. For these results, fractions 54, 57, 58, 60 and 64 were selected, which showed a reduction in bacterial growth, differing from each other as in E. coli and being more similar as in S. aureus and R. solanacearum.

Some compounds can inhibit growth more easily, which is the case of surfactin, which even at high doses only shows a reduction in bacterial growth (DE GIANI; ZAMPOLLI; DI GENNARO, 2021; LILGE et al., 2022), which is similar to the graphic results in figure 5. These compounds are used as adjuvants, due to their ability to destabilize the membrane by forming micelles (CERESA et al., 2023), a characteristic that is similar to detergents.

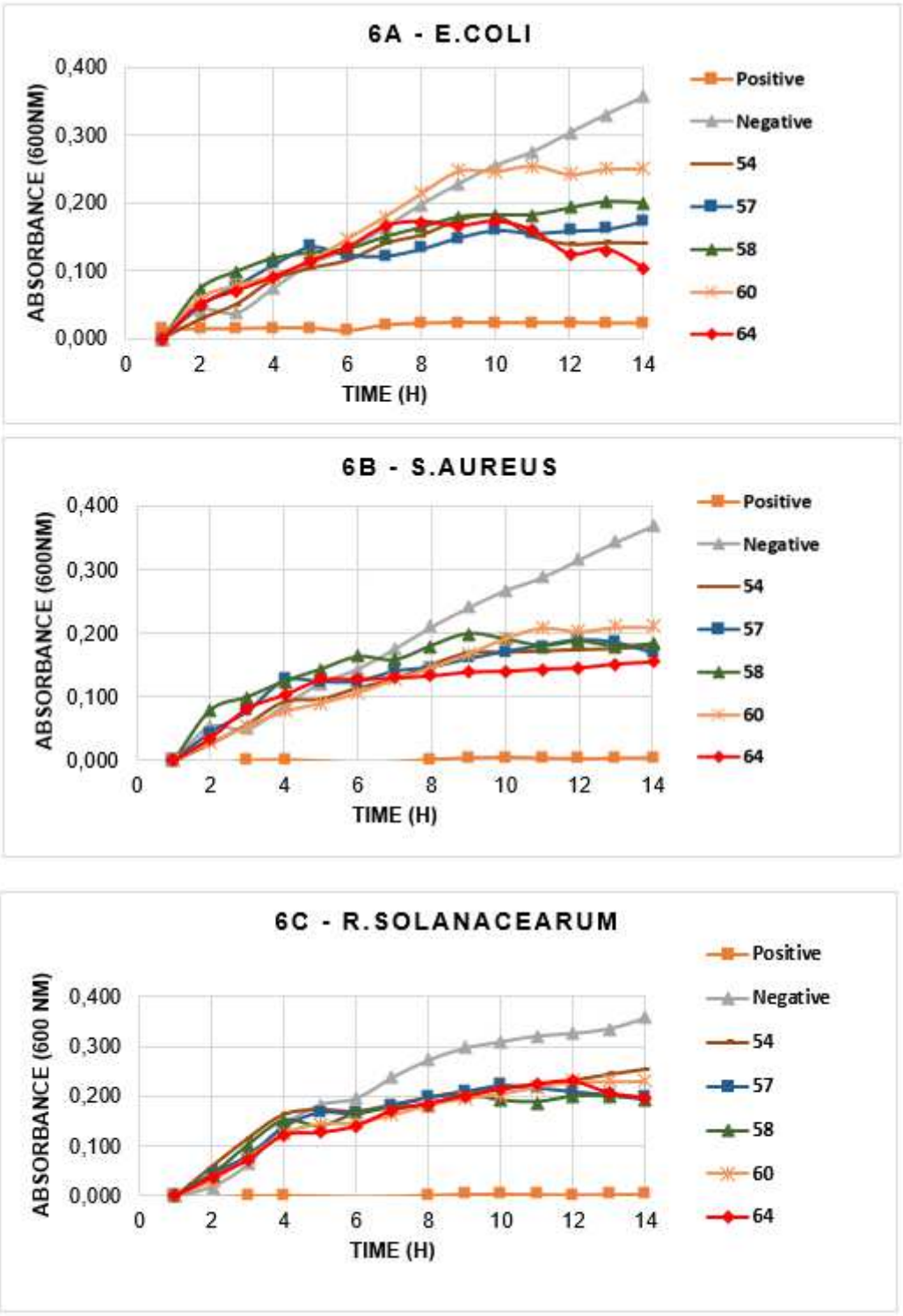
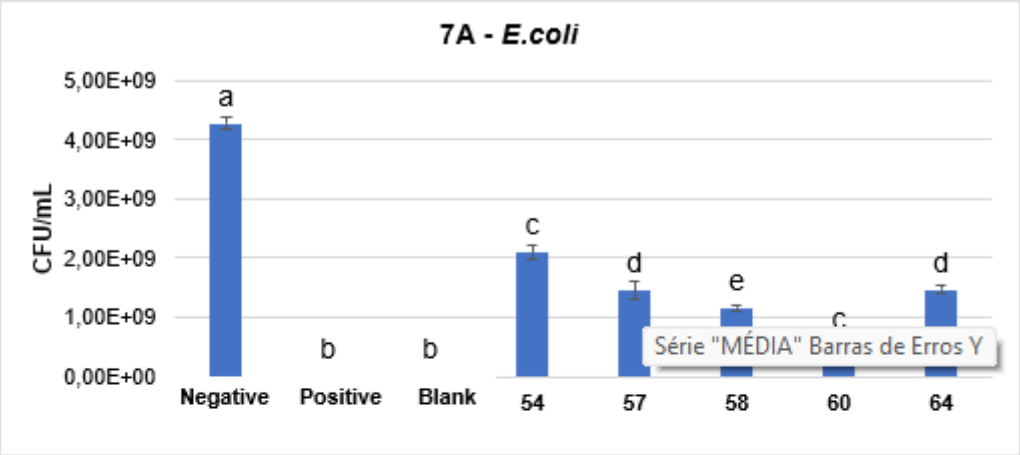
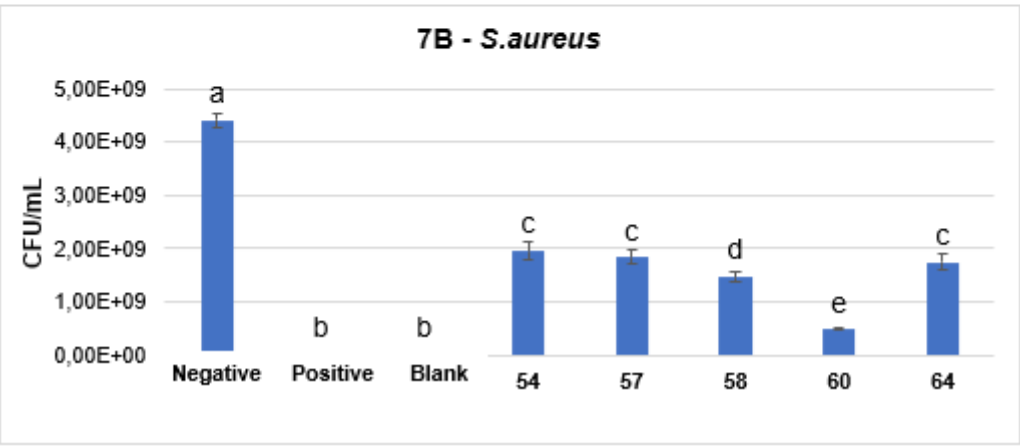


Figure 6: Bacterial growth curve subjected to treatment with fractions 54, 57, 58, 60, 64 of the chromatography of the ethanolic extract of C. legalis. Negative control: chloramphenicol 2.0 mg/ml / Positive control: bacterial growth without treatment / Numbers: chromatography fractions of the hydroalcoholic extract of pink Jequitibá





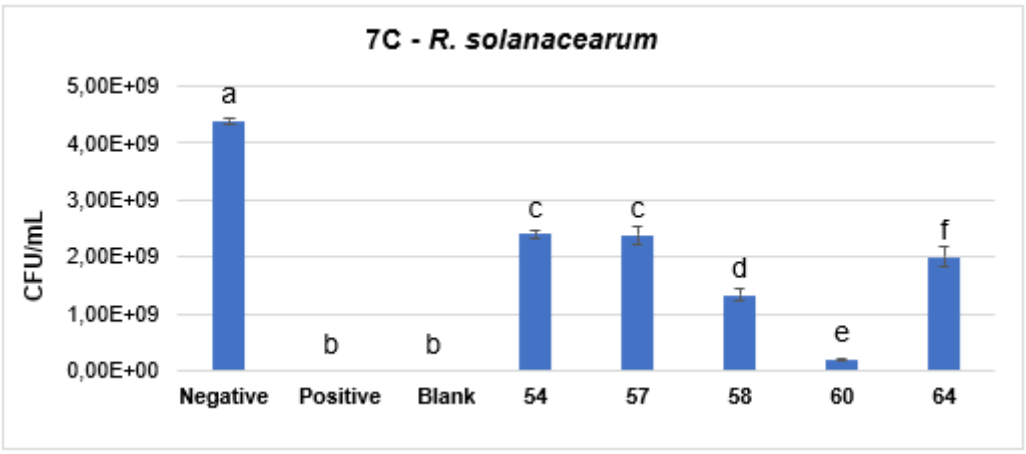


Figure 7: CFU/mL count of bacterial growth subjected to treatment of fractions 54, 57, 58, 60, 64, which showed lower bacterial growth than the control. Negative control: no treatment / Positive control: chloramphenicol 2.0 mg/ml / Blank: sterile culture medium. / Number: chromatography fractions.

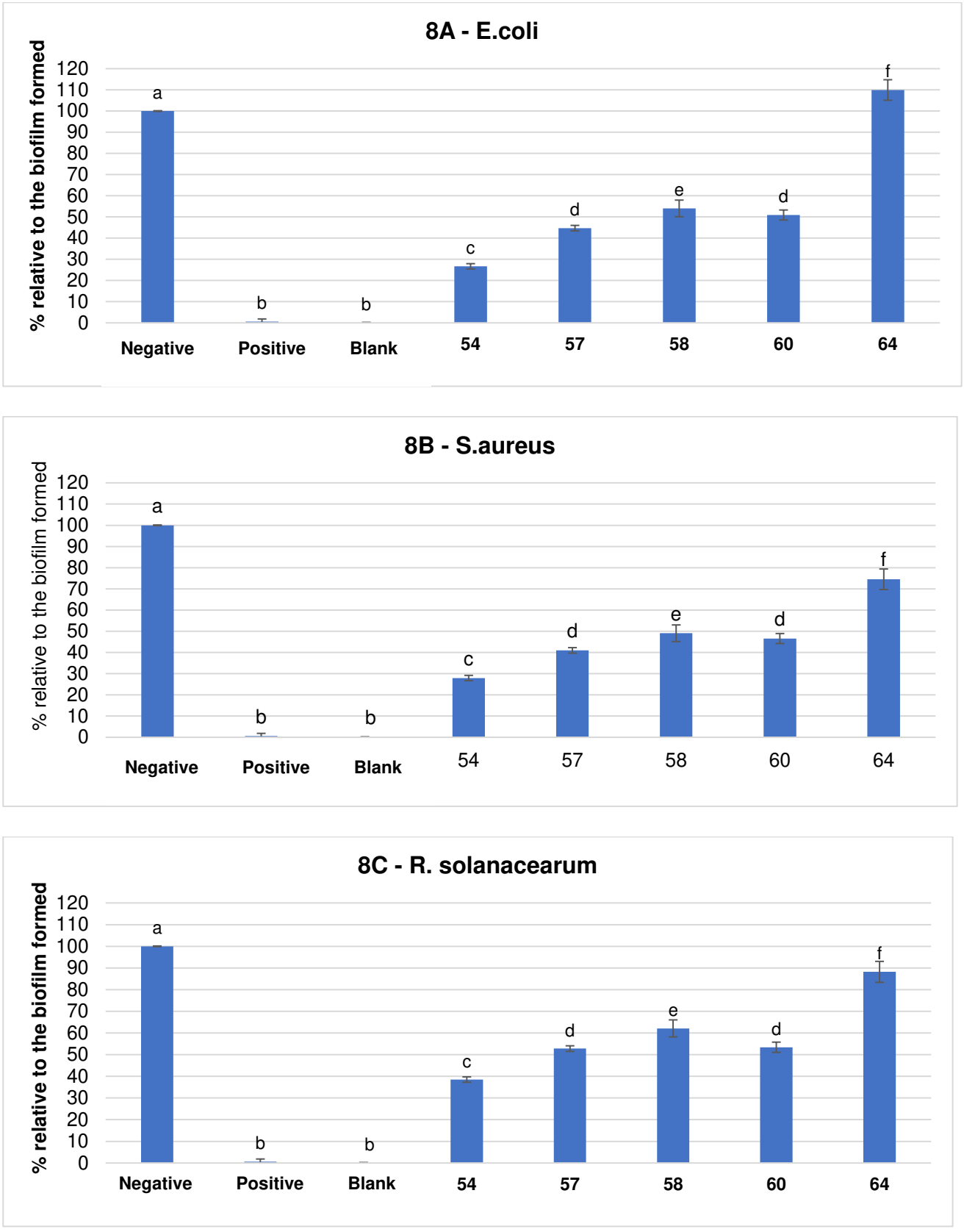


Figure 8: Inhibition of biofilm formation produced by bacteria subjected to treatment of chromatography fractions of C. legalis leaf extract. Negative control: no treatment / Positive control: chloramphenicol 2.0 mg/ml / Blank: sterile culture medium. / Number: chromatography fractions.

Analyzing figure 7, we observed a significant result for fraction 60, which stood out from the other aliquots by the Tukey test.

Since growth occurred only in a reduced form, the qualitative bactericidal effect would not be sufficient, so, as a form of guarantee, serial dilution of each well was performed to count the CFU (figure 7). The CFU count proved to be essential to reveal the growth inhibition effect, since at these concentrations the bactericidal experiment according to Mah and his collaborators (2014) would not be successful. Furthermore, the colony count corroborates the growth curve, showing that the number of colonies observed for each treatment in the curve is reliable for S. aureus and R. solanacearum, as is the case of fraction number 60, but showed differences in the case of E. coli, which in the curve graph showed similarity at the end of growth, but showed statistical differences in the CFU count.

For S. aureus and R. solanacearum, we observed that there was also a distinction, but with positive visibility, since fraction number 60 showed CFU values below the other fractions. The literature reports the use of resazurin (TWIGG, 1945) (RODRIGUES et al., 2024) to check the physiological conditions of the bacteria. If they are viable, they would metabolize resazurin (blue) to resorufin (pink). Since we still detect potentially active bacteria, this type of analysis would not be feasible, allowing a false interpretation of the viability of the bacteria. On the other hand, some chemical species may have a higher dosage to make the bacteria unviable, not allowing their cultivation in solid medium. Therefore, the CFU count was necessary for this experiment, to corroborate the fact that the fractions were diluted.

Figure 8 shows that all fractions reduced biofilm formation and differed statistically from each other. Fraction 54 stands out as it inhibited biofilm production more efficiently when compared to the other aliquots, while fraction 64 did not show antibiofilm capacity. The effects of fractions 57 and 60 were similar, reducing biofilm production by 48% and 47% for R. solanacearum, 60% and 54% for S. aureus and 56% and 50% for E. coli, respectively.

In addition to antibiotics, biosurfactants have the characteristic of inhibiting biofilm production, as they interact with the cell membrane, altering its stability on surfaces and making it difficult for it to adhere (DE GIANI; ZAMPOLLI; DI GENNARO, 2021; PATEL et al., 2021) as well as removing the biofilm already fixed in the locations (PATEL et al., 2021).

Putative compounds detected by GC-MS

All fractions were analyzed by GC/MS in an attempt to identify the bioactive compounds that could justify the antimicrobial activity. No significant differences were detected in the chromatographic profiles, only variations in the intensity value per peak between them. For mass spectrometry analysis, we selected the chromatogram of fraction 60, shown in Figure 9, because it exhibited the best antimicrobial activity.

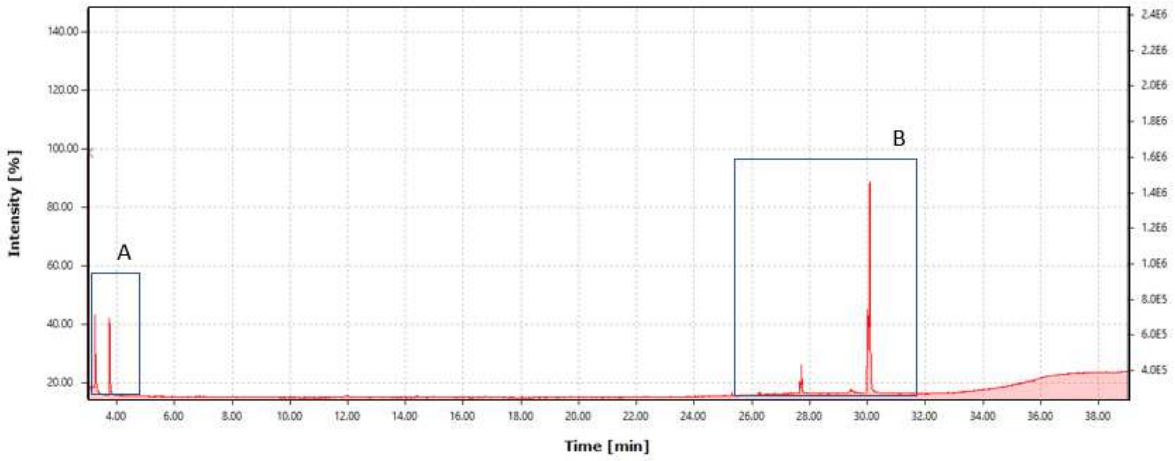
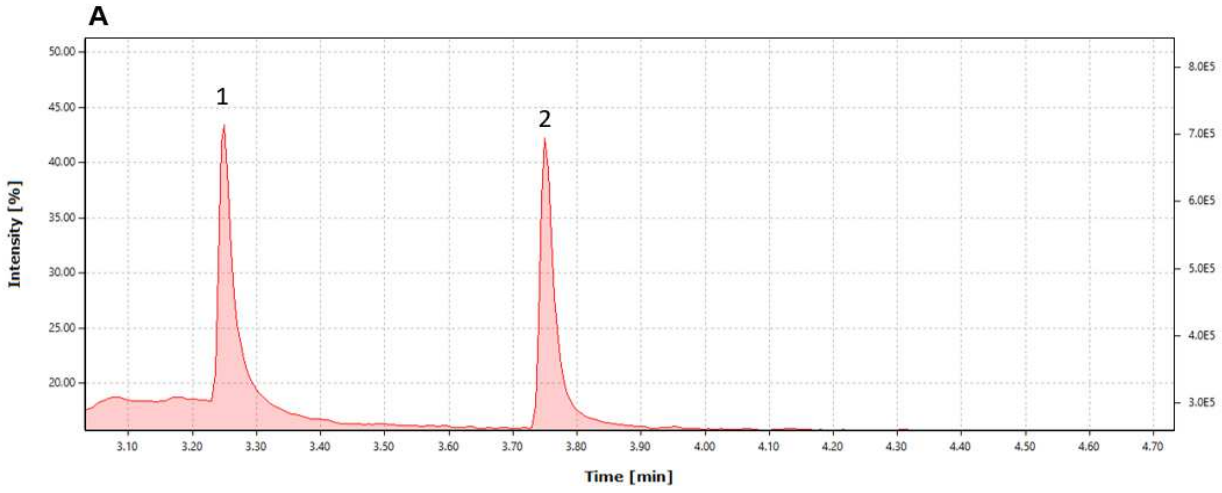


Figure 9: Chromatogram of fraction 60 of the purification process of ethanolic extract of pink jequitibá leaves.



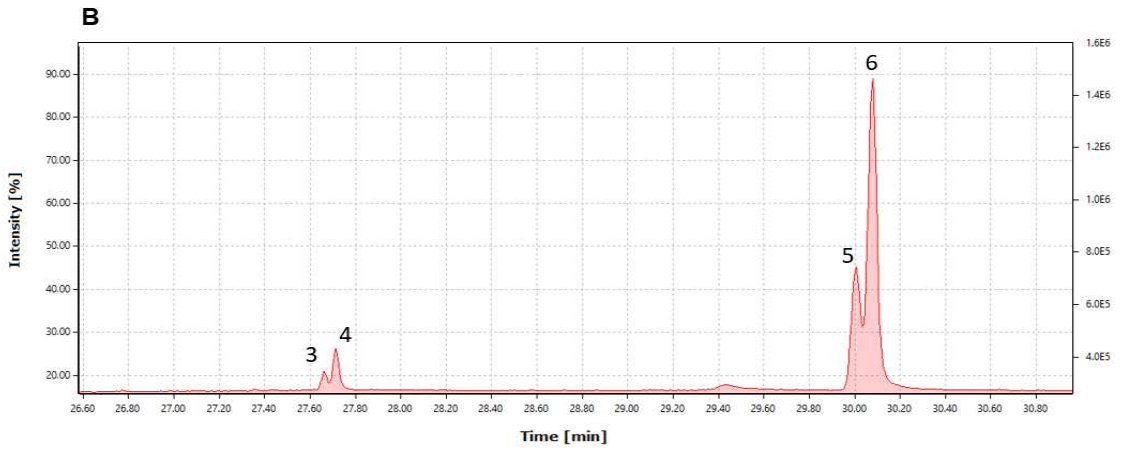


Figure 10: Enlarged chromatogram of fraction 60 revealing 6 total peaks / A: detection of 2 compounds / B: detection of 4 compounds.

The detected species had their peaks selected as shown in figure 10 and the mass spectrum aligned with the NIST14 library. Of the detected compounds, images 5 and 6 in figure 11 stand out, which may present antimicrobial characteristics. Structurally, their carbon chains are similar to stearic and oleic acids, respectively, with the addition of methyl esters. These structures are reported as promising and even candidates for antibiotics (CHOUDHARY et al., 2022), since in low concentrations they are excellent for reducing the growth of bacteria such as S. aureus (MRSA) and Pseudomonas sp (GANGAS; ALIYU; OYEWALE, 2021).

When combined with broad-spectrum antibiotics, they are good adjuvants and bring surprising results compared to a control when the antibiotic is used alone. The structure of compounds with the ester function is portrayed as biosurfactants that help to unbalance the bacterial membrane, causing rupture (LOURENÇO; DUARTE; RIBEIRO, 2024). In addition to their antimicrobial activity that prevents proliferation, biosurfactants have a history that continues to this day as compounds that prevent the adhesion of bacteria to surfaces, and therefore reduce the production of biofilm, as proposed by Souza et.al. (SOUZA et al., 2024) who introduced surfactants derived from soybean fatty acids (Glycine max) in mouthwashes and showed a significant reduction in the biofilm values synthesized by S. aureus from strains similar to that used in this work.

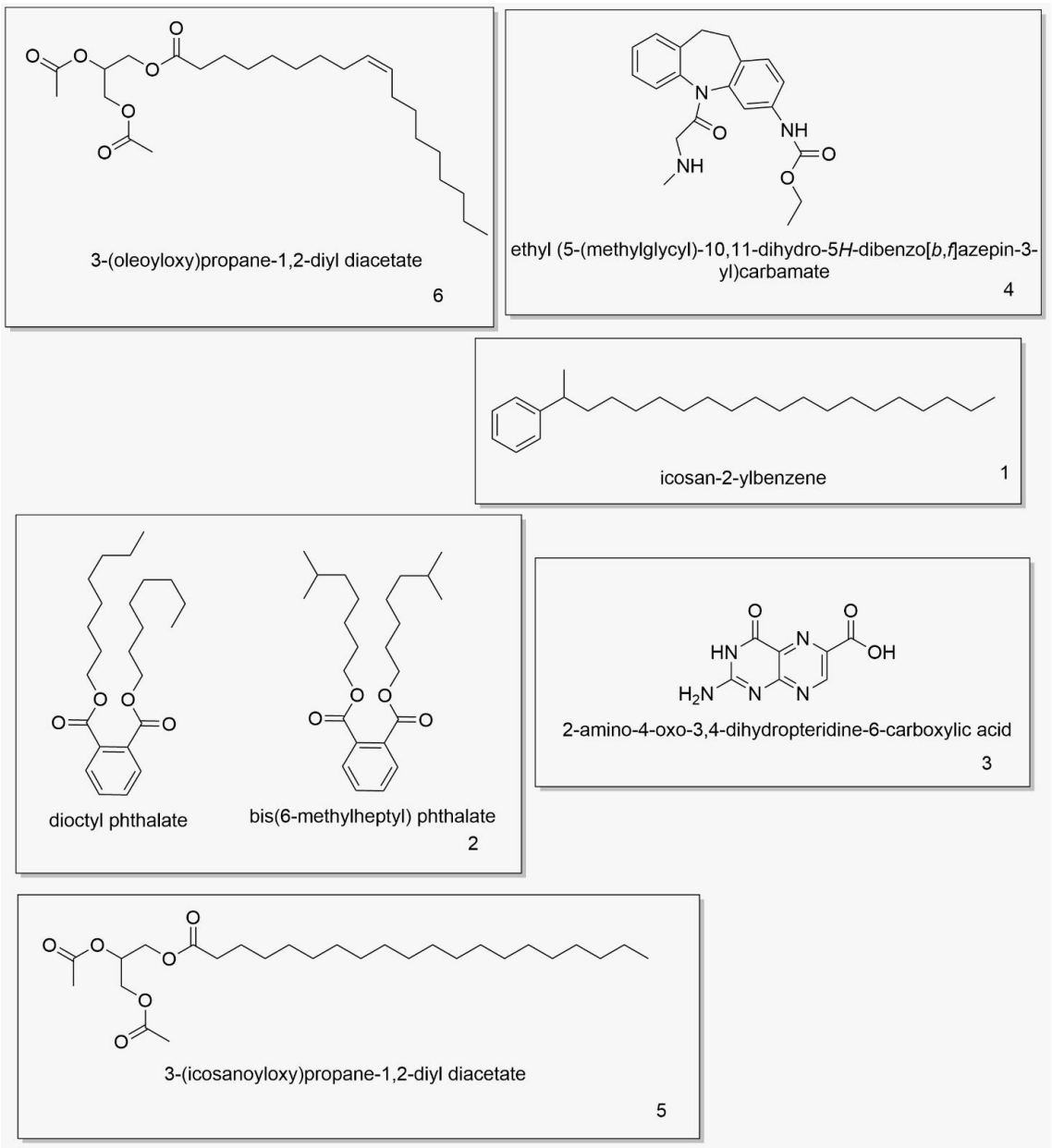


Figure 11: Compounds suggested in the ethanolic extract of jequitibá-rosa leaves by aligning the mass spectra with the NIST14 library.

Curcumins and coumarins are also reported in studies as amplifiers of antimicrobial activity, in addition to collaborating as anti-inflammatory, immunomodulatory (EL-ANSSARY et al., 2021) and anticâncer (RAHAMOUZ-HAGHIGHI et al., 2022) agents, similar structures as we detected in image 2 of figure 11. These two structures were considered for specimen 2 in the GC, as both presented 85% similarity with alignment in the mass spectra library.

Table 2: Data from the chromatographic process and separation of compounds that were detected and approximated by alignment in the ethanolic extract of *Cariniana legalis*

Compound	Retention time (min)	% similarity (NIST 14)
1	3,267	81%
2	3,755	85%
3	27,619	62%
4	27,715	63%
5	30,005	82%
6	30,165	80%

Another class worth highlighting are purines with modified characteristics, with linked rings such as images 3 and 4, which were detected with 62 and 63% similarity with the library. Their nitrogenous structure suggests that they came from metabolic pathways of nucleotide synthesis that were modified. These structures are studied as synthetic antimicrobials and may have constituent amide structures described by Singh et. al. (2022).

Conclusion

As evidenced, we can see that the ethanolic extract of *Cariniana legalis* leaves has antimicrobial properties and reduces the production of bacterial biofilm. The study reveals the presence of unreported chemical compounds present in plants, whose structures confirm the function reported in this work.

Declaration of conflict of interest

On behalf of all authors, the corresponding author states that there is no conflict of interest.

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