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IN VITRO ACTION OF PLANT EXTRACTS ON ENTEROCOCCUS FAECALIS

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ABSTRACT

Enterococci faecalis plays a significant role in diseases in clinical dental practice, especially as a causative agent of endodontic and soft tissue infections. The constituents of medicinal plants can be used in association with medicines, as raw material, or directly as pharmacologically active compounds and offer an alternative or adjuvant in antimicrobial treatment, and can be a useful resource in dentistry. The aim of the present study was to evaluate the inhibitory potential and interaction with amoxicillin of medicinal plant extracts

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Revista ARACÊ
ISSN: 2358-2472

on E. faecalis. Two strains of E. faecalis, ATCC 29.212 and ATCC 51.299, were used to determine the percentage of bacterial growth inhibition, interaction of antimicrobials with plant extract, and action on biofilm formation. The ethanolic extracts of Lantana camara, Lippia macrophyla, Genipa americana, and hexane extracts of Hypericum connatum and H. caprifoliatum were used in concentrations ranging from 31.1 to 1000 µg/mL. For the tests of interaction between plant extract and amoxicillin, the checkerboard method was used with concentrations of L. camara extract from 16 to 1000 µg/mL and amoxicillin from 0.16 to 10 µg/mL. To verify the action on biofilm formation, the violet crystal method was used. All the extracts tested inhibited the growth of E. faecalis varying according to strain and extract. For the ATCC 29.212 strain, all extracts presented results significantly lower than the mean value of chloramphenicol and, at the concentration of 500 µg/mL of the extracts, comparable to the mean value of amoxicillin. For ATCC strain 51,299, no extract demonstrated action comparable to chloramphenicol or amoxicillin. Regarding the action on the formation of the biofilm, it was observed that the extract of L. camara at a concentration of 1000 µg/mL does not interfere with the action of amoxicillin. It is concluded, therefore, that extracts of plant species of the Verbenaceae, Hypericaceae and Rubiaceae families are promising as inhibitors of E. faecalis growth in vitro, and further research is recommended to establish whether or not these extracts are effective in their clinical application in the search for alternative treatments in oral disease.

Keywords: Medicinal Plants. Enterococcaceae. Oral Alterations. Antimicrobials.

Revista ARACÊ
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INTRODUCTION

Pathologies of the oral cavity, especially periodontal diseases and dental caries, are considered a Public Health problem, capable of causing negative impacts on the community and an increase in the demand for treatment and swelling of the demand for health centers (Spezzia, 2015). According to the World Health Organization (WHO), almost half of the world's population suffers from oral diseases, affecting about 3.5 billion people. Among the main oral problems, periodontal diseases and caries represent a large part of these occurrences. According to world data, even today, 43.75% of the world's population has cavities (WHO, 2022).

In this context, traditional dental treatment for bacterial infections focuses primarily on removing the cause and, as an ancillary measure, on the use of antibiotics. The administration of antimicrobials can act both in the elimination and inhibition of bacterial growth, or be used prophylactically in patients with systemic disorders and/or at risk of developing bacterial endocarditis (Almeida et al. 2014). However, the excessive and/or inappropriate use of antibiotics can negatively compromise the patient's clinical response, which can increase hospitalization costs, in addition to contributing to the appearance of multi-resistant bacteria to antimicrobials. This means that drugs that were previously effective in treating infections become less or completely ineffective, making it difficult to control and treat these infections (Brigantini et al., 2016).

A promising approach to combat bacterial resistance is the use of natural products, especially those derived from medicinal plants, that contain bioactive compounds with antimicrobial potential against resistant strains (Isola, 2020; Moro et al., 2018) and related to oral pathologies, such as S. mutans (Silva; de Oliveira; Coelho, 2024). These compounds may have different mechanisms of action than traditional antibiotics, offering an effective alternative. In addition, the development of natural products to treat oral infections stands out for its low cost, lower risk of adverse effects, and proven biocompatibility, making it a viable option in alternative medicine (Da Silva Sales et al., 2023).

Natural and herbal compounds used in oral health become allies in preventive and curative programs, preventing bacterial growth, adhesion, and colonization, as well as anti-inflammatory, anti-hemorrhagic, and anesthetic action (Domingues et al., 2021). In Dentistry, for example, the insertion of natural products can help control the growth of dental biofilm. It can be highlighted that the bioactive components of some natural products have the potential to prevent and treat periodontal disease, helping to reduce the subgingival

Revista ARACÊ
ISSN: 2358-2472

microbial community associated with the formation of gingivitis and oral candidiasis (Sakagami et al., 2018; Brazil, 2022). Reports in the literature demonstrate that both isolated substances and plant extracts may have potential action on E. faecalis. Of note are the experimental studies with an extract rich in ent-kauneroic acid (KAMg) obtained from Mikania glomerata, guaco (Moreira et al., 2016); pericarp extract from Garcinia mangostana (mangosteen) (Janardhanan et al., 2017); flavonoids by Gutiérrez-Venegas et al. (2019) and the methanol extract of Rumex nervosus, a plant used in herbal medicine in Saudi Arabia (Al-Farhan et al., 2022).

The results of the action of plant extracts have been promising even in resistant strains of E. faecalis. The extract of Psidium guajava, for example, by the action of phenolic compounds and flavonoids, inhibits the growth of E. faecalis by compromising the integrity of the cell membrane (Da Silva Sales et al., 2023) and the extract of Casearia sylvestris by the presence of caseary acid can also interfere with the cell structure of the bacterium, causing its inhibition and possible death (Vicari et al., 2022). These extracts are considered promising alternatives due to their efficacy even against resistant strains, expanding treatment options in the dental context.

Plants of the Verbenaceae, Hypericaceae and Rubiaceae families are recognized in folk medicine and, scientifically, for their bioactive components. Of these plants, studies have been carried out with extracts of the genera Lippia, Lantana, Genipa and Hypericum, with promising results for the activity against different microorganisms. Thus, in view of the scarce reports of antimicrobial activity on E. faecalis, this study aimed to verify the inhibitory action and biofilm formation of the crude extracts of the plant species Lantana camara, Lippia macrophylla, Genipa americana, Hypericum connatum and H. caprifoliatum on two strains of E. faecalis.

MATERIAL AND METHODS

MICRO-ORGANISMS

For the antimicrobial activity tests, the strains of E. faecalis (Andrewes and Horder) Schleifer and Kilpper-Balz ATCC 51.299 and E. faecalis (Andrewes and Horder) Schleifer and Kilpper-Balz (ATCC 29.212) were used. Staphylococcus aureus (ATCC 29.213) was also used to control biofilm formation. The strains were kindly provided by Acassia Lippi of the Fleury Laboratory, Belo Horizonte, Minas Gerais, Brazil.

Revista ARACÊ
ISSN: 2358-2472

The bacteria were confirmed for morphology (Gram) and phenotypic characteristics (catalase, oxidase and susceptibility to antimicrobials) and stored in the Microbiology Laboratory of the State University of Santa Cruz in Lignière medium. For use, these were reactivated in Brain Heart Infusion (BHI) broth 24 hours before the experiments.

In order to verify the susceptibility of the selected microorganisms, an antimicrobial susceptibility test described by BrCast (2021) was carried out against antimicrobials commonly used in dental clinics, such as amoxicillin (10 µg; CECON, São Paulo, Brazil), and vancomycin (10 µg; CECON, São Paulo, Brazil) through the agar diffusion method, according to the standard norms of BrCAST (2021) with modifications. For this, the inoculums were standardized to 0.5 on the MacFarland scale and spread entirely in a Petri dish with Muller-Hinton agar (Kasvi, Paraná, Brazil) plus 5% of human blood with anticoagulant. After drying the inoculum on the plate, the discs containing antimicrobials were placed on the agar aseptically. The plate was incubated at 37 °C for 18 h in microaerophilia, after which the diameter of the inhibition halo was measured in millimeters and analyzed for the recognized standards for each antimicrobial for sensitive (S) or resistant (R).

SELECTED PLANT EXTRACTS

The species collected in the state of Bahia, between the cities of Ilhéus and Itacaré, Lippia macrophylla (HUESC21,065), Lantana camara (HUESC21,064) and Genipa americana (13,923), were identified by the botanist and registered in the herbarium of UESC (State University of Santa Cruz). Projects involving extracts from these plants are registered in the Brazilian Associated Genetic Heritage and Traditional Knowledge Management System (SISGEN) under numbers A049687, A2153BD and A7C08DC, respectively.

The ethanolic extracts of Lantana camara and Lippia macrophylla and the branches of Genipa americana were obtained from dried and crushed material, macerated in PA ethanol in three 24-h cycles and evaporated until dry mass was obtained, which was later stored at 4-8ºC, as described in Silva (2016) and Codignoto et al. (2017). The ethanolic extracts were also solubilized in dimethyl sulfoxide at 100 mg/mL and stored at -20°C until use.

The species of H. connatum and H. caprifoliatum were collected in 2006, in the state of Rio Grande do Sul, identified by botanist and exsicata stored in the herbarium of the

Revista ARACÊ
ISSN: 2358-2472

Federal University of Rio Grande do Sul (ICN/UFRGS). The hexane extracts were obtained by the Pharmacognosy group of UFRGS, led by prof. Gilsane Lino Von Poser and are described in Conceição et al. (2014). Access to the plants was obtained from IBAMA (Brazilian Institute of the Environment and Renewable Natural Resources) registration (n. 003/2008; Protocol 02000.001717/2008 - 60) and are registered in Sisgen under number A97E6BC.

EVALUATION OF THE PERCENTAGE OF MICROBIAL GROWTH INHIBITION

To determine the percentage of microbial growth inhibition of plant extracts, the technique described in Söderling et al. (2008) was adopted, with modifications. The extracts solubilized in DMSO were diluted in BHI and tested at concentrations of 0.031, 0.062, 0.125, 0.25, 0.5 and 1 mg/mL. From the previously prepared dilutions, 90 µL of the treatments at the respective concentrations were distributed in a 96-well microplate, and then 10 µL of bacterial inoculum was added, which was adjusted to 1.0 on the Mc Farland scale, so that all the microplate wells accommodated a final volume of 100 µL.

Each test was performed in triplicate and repeated three times. As positive controls, the antibiotics chloramphenicol at 50 µg/mL and amoxicillin at 0.32 µg/mL (cut-off point value BrCast, 2023) were used, as negative controls samples of the extracts described at the lowest concentration tested, the bacterial inoculum, in addition to wells containing only BHI. The microplates were stored at 36±1 °C. After 24 hours of bacterial growth, a spectrophotometer (EZread, Analytical) reading was performed at 450 nm wavelength. To evaluate the percentage of inhibition of the extracts, the following formula was used: % Inhibition = χDOt·100/χDObac-100. Where: DOt450nm = optical density at wavelength of 450 nm of the treatments; DObac450nm = optical density at 450 nm wavelength of bacterial inoculum.

Also, 10 µL of the dye resazurin (Aldrich Chemistry) at 0.01% final concentration was applied in each of the wells of the microplate and waited 1 to 2 h to verify bacterial viability. The bactericidal action was evaluated according to blue or pink coloration, characterizing the absence or presence of bacterial growth after 24 h, respectively.

The results were stored in an Excel spreadsheet and the graphs were prepared using the GraphPad Prism version 5 (2007) program. The inhibition efficacy of plant extracts was compared to chloramphenicol and amoxicillin by the Oneway ANOVA

Revista ARACÊ
ISSN: 2358-2472

statistical tests followed by the Tukey test and the t-test. For this purpose, a confidence interval of 95% ($p<0.05$) was considered.

EVALUATION OF THE COMBINATION OF EXTRACT AND AMOXICILLIN BY THE CHECKERBOARD METHOD

To evaluate the effect of the combination between the plant extract and antimicrobial, the checkerboard method described in Fernández-Cuenca et al. (2003) was used, with modifications.

For the test, we chose the L. camara extract at the highest concentration tested (1000 µg/mL) due to the availability of the extract in the laboratory.

Amoxicillin was chosen because it is the antimicrobial of choice in the dental clinic. The concentrations of amoxicillin chosen were less than 10 µg/mL according to the cut-off (10 µg/mL) published by BrCast (2023). Also, by the broth microdilution technique, the minimum inhibitory concentration (MIC) of amoxicillin was established for the two strains of E. faecalis in use in the experiment, obtaining a value of 0.31 µg/mL for both strains.

To perform the test, 45 µL of each twice-concentrated substance was added to the lines and columns of the 96-well flat-bottom plate. In the columns, concentrations of amoxicillin were added at final concentrations of 0.01 to 10 µg/mL and in the rows, 45 µL of L. camara from 15.6 to 1000 µg/mL were added.

The bacterial inocula of the two E. faecalis strains ATCC 51.299 and ATCC 29.212 were adjusted to 1.0 on the McFarland scale and 10 µL of them were distributed in all wells. After 24 hours of incubation at 36.5 °C, in microaerophilia, 10 µL of 0.1 % resazurin was added to each orifice. After 1 to 2 hours, the MIC was determined for each substance in the combination between them. To interpret the interaction, fractal inhibitory concentrations (FIC) were calculated using the formula described in Tong et al. (2006): FIC(A) = MIC (A) in combination/MIC (A) alone; CIF (B) = MIC (B) in combination/MIC (B) alone; ΣCIF = CIF (A) + CIF (B). The results were interpreted according to the ΣCIF value: ΣCIF – ≤ 0.5 corresponds to a synergistic effect; ΣCIF – ≤ 0.75 corresponds to a partially synergistic effect; ΣCIF – > 4 corresponds to an antagonistic effect; ΣCIF between 0.75 and 4 corresponds to indifference.

Revista ARACÊ
ISSN: 2358-2472

INTERFERENCE TEST OF BIOFILM FORMATION

In the interference assay in the formation of the biofilm, the violet crystal method was used. To perform the technique, bacterial inoculums were adjusted to 0.5 on the McFarland scale. Then, 96-well plates were filled with 100 µL of the different treatments at the final concentration to be tested. The treatments consisted of amoxicillin at 0.31 µg/mL and ethanolic extract of L. camara at 1000 µg/mL. Afterwards, 100 µL of the inoculums were added to each orifice. As a control for biofilm formation, S. aureus was used without antimicrobial and with gentamicin at a concentration of 10 µg/mL and sub-MIC of 0.31 µg/mL (BrCast, 2021).

The plates were kept for 48 h in an incubator at 36.5 ºC, in microaerophilia, and after formation and maturation of the biofilm, the supernatant medium was carefully removed and the wells were washed with sterile pH 7.4 saline phosphate buffer (PBS) to remove the planktonic cells. For the fixation of the benthic cells, 200 µL of methanol were added to each culture well and allowed to act for 15 min.

After methanol removal, the biofilm was left to dry at room temperature and 200 µL of 1 % violet crystal was added to each hole. After 5 minutes of action, the dye was removed and the biofilm was gently washed with PBS three times. Finally, the biomass was solubilized with 100 µL of 96º ethanol to release and homogenize the contents of the wells. After 10 minutes, a spectrophotometer reading was performed at a wavelength of 570 nm.

The results were stored in an Excel spreadsheet and the graphs were prepared using the GraphPad Prism v. 5 (2007) program. The influence of plant extracts with or without the combination with amoxicillin on biofilm formation analyzed through the statistical tests Oneway ANOVA followed by Tukey test and t-test. For this purpose, a confidence interval of 95% ($p<0.05$) was considered.

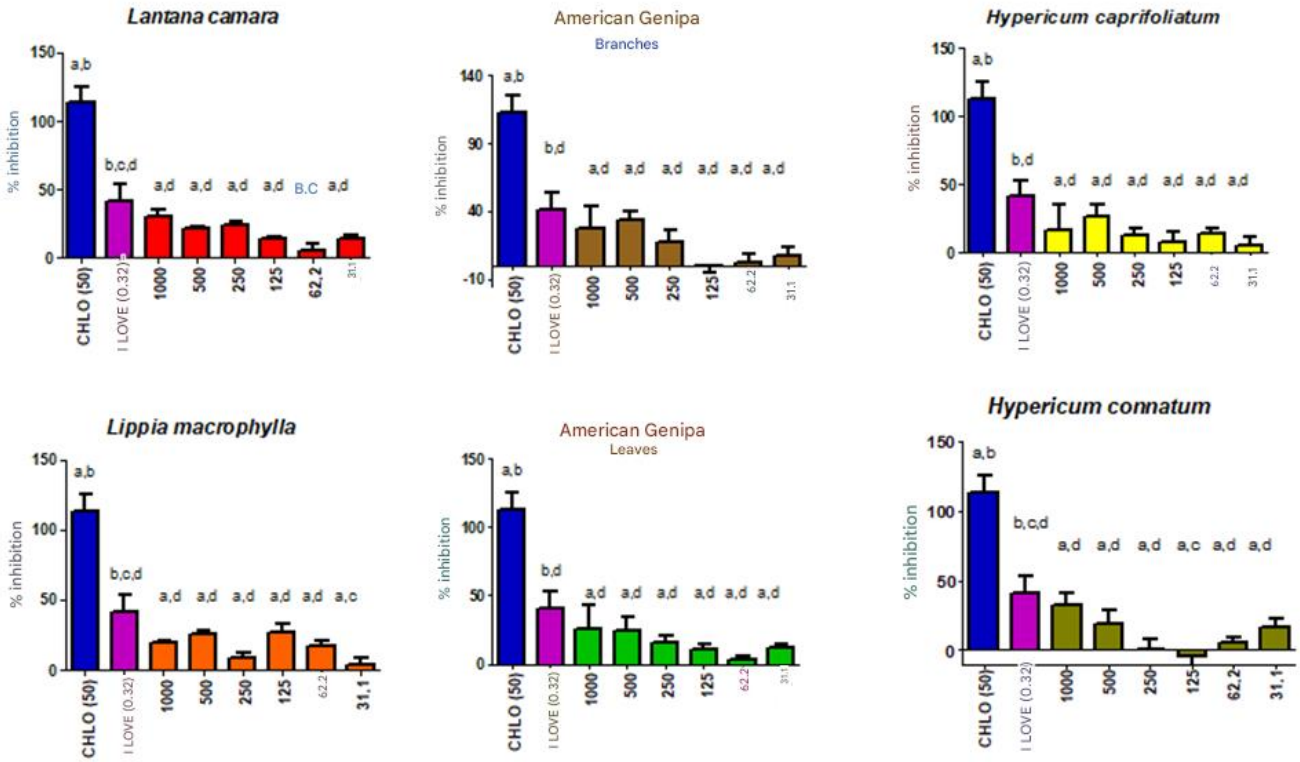
RESULTS AND DISCUSSION

The search for natural alternatives in the treatment of infections caused by E. faecalis has gained prominence in dentistry, especially due to the growing bacterial resistance to traditional antibiotics. The existing literature demonstrates that plant extracts have a broad spectrum of antimicrobial activities and can be valuable alternatives for dental treatment. Thus, in this work, we observed the inhibitory effect with bacteriostatic characteristic of plant extracts from the Atlantic Forest on E. faecalis.

Revista ARACÊ
ISSN: 2358-2472

In this study, for both strains of E. faecalis, the extracts showed a bacteriostatic effect, seen by their viability after 24 hours. Regarding the percentage of inhibition, for the E. faecalis strain ATCC 29.212 (Fig. 1), all extracts presented significantly lower results ($p<0.01$) than chloramphenicol (113.1 % ±24.6). However, in comparison with Amoxiclina, whose percentage of inhibition was (40.9 % ±24.52), the ethanolic extract of L. camara (30.2 % ±11.02) and the hexane extract of H. connatum (32.42 % ±17.07), both at a concentration of 1000 µg/mL, and the ethanolic extract of the branches of G. americana (33.21 % ±13.12) at 500 µg/mL were comparable in percentage of inhibition to this antimicrobial.

Figure 1. Percentage of inhibition of chloramphenicol, amoxicillin and plant extracts on the strain of E. faecalis ATCC 29.212 by the broth microdilution technique

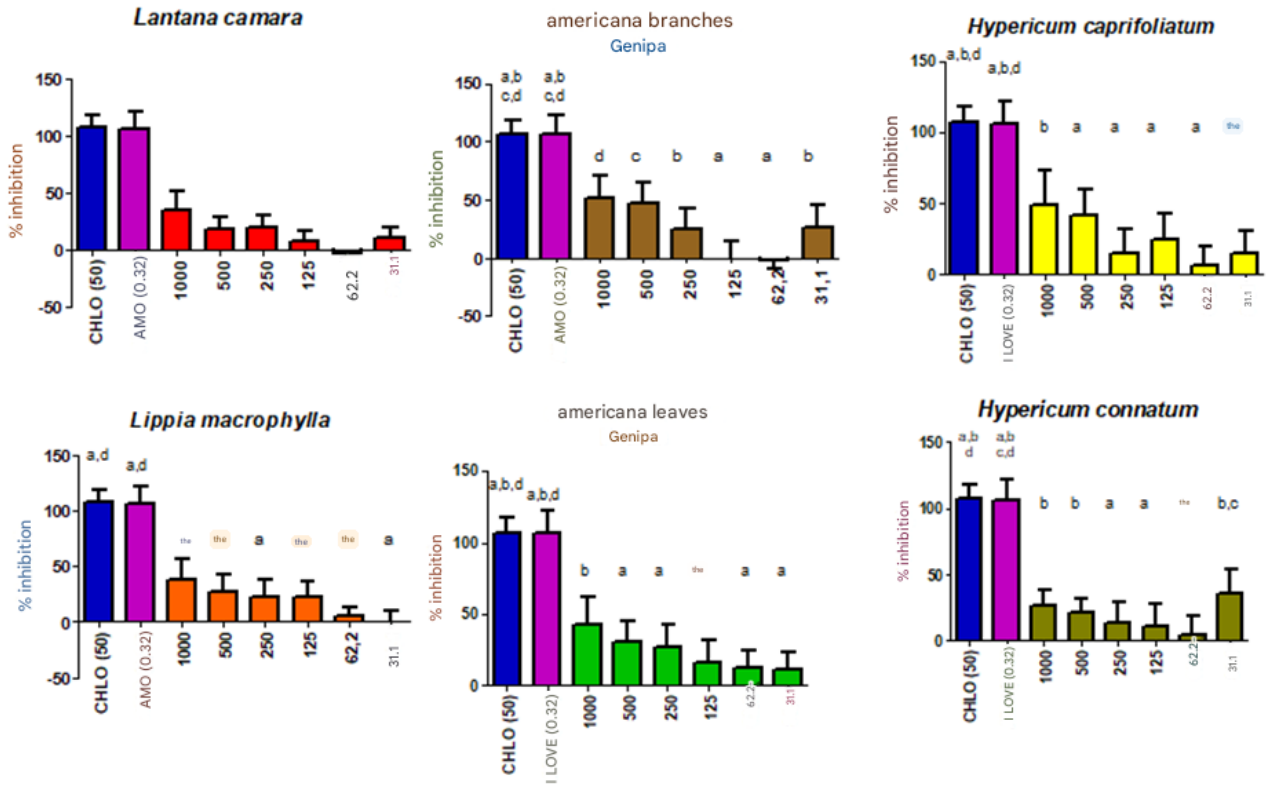


Legend: CHLO – chloramphenicol at 50 µg/mL; AMO – amoxicillin at 0.32 µg/mL. Note: Comparison using the t-test at a 5% significance level, in which a: $p<0.001$; b: $p<0.05$; c: $p<0.5$; d There is no significant difference between treatments.

For the ATCC 51.299 strain of E. faecalis, in addition to the same bacteriostatic effect, no extract demonstrated action comparable to the two antimicrobials used. All samples showed mean inhibition percentages below 50 % at all concentrations tested (Fig. 2).

Revista ARACÊ
ISSN: 2358-2472

Figure 2. Percentage of inhibition of chloramphenicol, amoxicillin and plant extracts on the strain of E. faecalis ATCC 51.299 by the broth microdilution technique



Legend: CHLO – chloramphenicol at 50 µg/mL; AMO – amoxicillin at 0.32 µg/mL. Note: Comparison using the t-test at a 5% significance level, in which a: p<0.001; b: p<0.05; c: p<0.5; D: There is no significant difference between the treatments.

Studies such as those by Tonino-Rivera et al. (2016) and Oliveira et al. (2018) have shown that species of the genus Lippia have potent antimicrobial properties, being effective against several bacterial strains including Streptococcus mutans, an important agent in oral health. Silva (2016) highlighted that extracts of Lantana camara and Lippia macrophylla showed significant inhibition of porcine herpesvirus type 1, suggesting a broad spectrum of antiviral activity. With the present work, another opportunity is opened for the study of the therapeutic possibilities against microorganisms of plants of the genus Verbenaceae.

As for the species of the genus Hypericum, the unprecedented result of the hexane extract of H. connatum on the strain ATCC 29.212 stands out, whose action was comparable to that of the antimicrobial amoxicillin. The present study reiterates the antibacterial potential of this plant species, considering that a previous study conducted by Fratianni et al. (2013) already demonstrated broad-spectrum antibacterial activity and inhibition of bacterial quorum sense regulation by polyphenol-rich ethanolic extract.

The biological potential, including antimicrobial activity, of the Genipa americana species has been well reported in the literature and synthesized in a review by Assis et al.

Revista ARACÊ
ISSN: 2358-2472

(2023). Codignoto et al. (2017), for example, demonstrated the bactericidal action of G. americana extract on E. coli and bacteriostatic action of fruit extract on S. aureus. It should be noted that, in the present study, we present the bacteriostatic action of the ethanolic extract of the branches of this plant on E. faecalis, expanding the spectrum of action of this extract. In view of the result similar to that of S. aureus, a differentiated action associated with the type of bacterial wall is suggested, instigating further studies on the mechanism of action of these plant extracts.

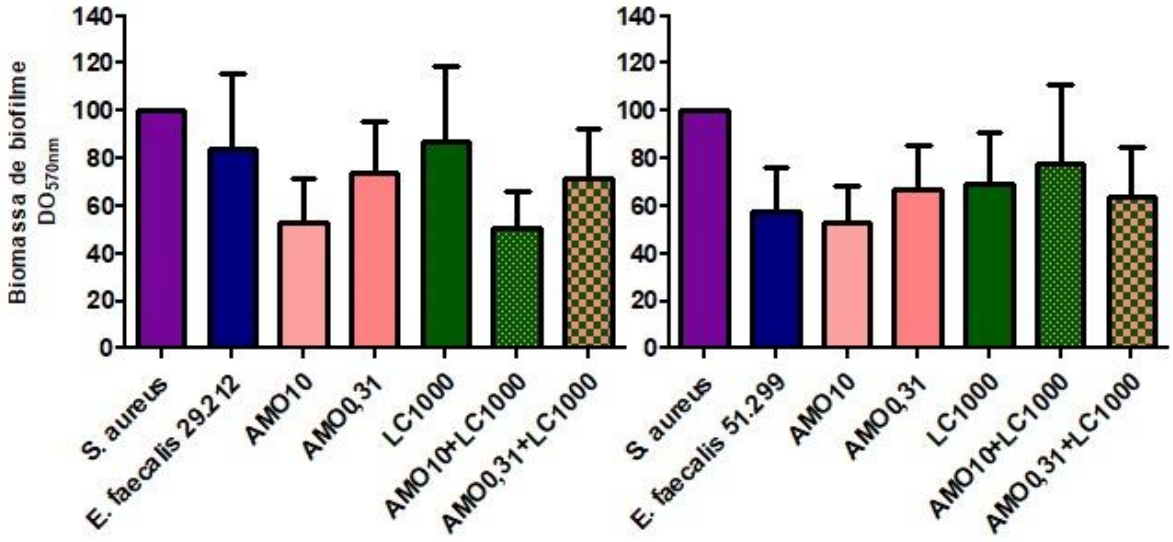
For the tests of interaction between plant extract and antimicrobial, for both strains, it was observed that the minimum inhibitory concentration (MIC) of L. camara extract was 16 µg/mL when in conjunction with amoxicillin and the MIC of this antimicrobial when in combination with L. camara extract was not altered, remaining at 0.31 µg/mL. Thus, regarding the combined effect of L. camara with amoxicillin, it was observed that the ΣCIF was 1.016, indicating as a result, an indifferent effect. It is noteworthy here that if, on the one hand, there is no synergistic or antagonistic effect on amoxicillin, an optimization of the effect of the plant extract is obtained with the possibility of using a smaller amount of extract in future studies, in the presence of this antimicrobial.

Regarding biofilm formation, it was observed that the strain ATCC 29.212 was able to form a biofilm mass comparable to the biofilm-forming strain of S. aureus ATCC 29.213 (84.12 %) (Fig. 3). The ATCC 51.299 strain, resistant to Vancomycin, formed 42.61% less biomass than the biofilm-forming S. aureus strain. Observing the action of amoxicillin on the biofilm formation of the two strains of E. faecalis, although without statistical significance (p=0.144), at a concentration of 10 µg/mL, the biofilm biomass of the strain ATCC 29.212 showed an important reduction in biomass of 31.37%. For the ATCC 51,299 strain this reduction was smaller at the same concentration, being only about 5%. In the case of treatment with the minimum inhibitory concentration (0.31 µg/mL), an increase in biomass of 9.19 % is marked.

Regarding the treatment of E. faecalis strains with L. camara ethanolic extract at a concentration of 1000 µg/mL, an increase in bacterial biomass was observed, which was more intense for the ATCC strain 51,299 (11.99 %) than for the ATCC strain 29,212 (3.04 %). In the associated treatment of L. camara extract with amoxicillin, it was observed that the extract did not interfere with the action of amoxicillin for the ATCC 29,212 strain. Meanwhile, for the ATCC 59.212 strain, a slight increase (10.58 %) was observed in the production of biomass.

Revista ARACÊ
ISSN: 2358-2472

Figure 3. Evaluation of the action of ethanolic extract of L. camara on biofilm formation in strains of E. faecalis ATCC 29.212 and ATCC 51.299



Legend: S. aureus: Staphylococcus aureus ATCC 29.213; E. faecalis 29.212: Enterococcus faecalis strain ATCC 29.212; AMO10: treatment of E. facealis with amoxicillin at 10 µg/mL; AMO0.31: amoxicillin at 0.31 µg/mL; LC1000: ethanolic extract of Lantana camara at 1000 µg/mL.

The reviewed studies on biofilms bring important contributions, elucidating both the factors that influence the formation of these biofilms and the interactions with the host. A point of convergence between the studies is the innate ability of E. faecalis to form biofilms in diverse environments and surfaces, regardless of the presence of specific proteins or genes, as observed by Kristich et al. (2004) and Guerreiro-Tanomaru et al. (2013). Both studies highlighted that biofilm formation occurs on a variety of substrates and that external factors, such as the type of surface and the growing environment, can influence the organization and growth of these biofilms. The study by Kafil et al. (2016) shows that exposure to gentamicin induces a significant increase in the formation of biofilms and in the expression of genes associated with colonization factors, which highlights the direct impact that antimicrobial agents or, in the case of the present study, plant extracts can have on the behavior of the pathogen.

CONCLUSION

In the present study, of the plant products studied, we highlight the action of L. camara, L. macrophylla and H. connatum extracts on E. faecalis. The extracts of these plants showed a promising potential as antimicrobial agents, with variations in their efficacy according to different strains of E. faecalis and combination with the antibiotic amoxicillin. However, although the results were promising, the need for further optimization and

Revista ARACÊ
ISSN: 2358-2472

investigations are needed. The interaction of extracts with other antimicrobials for use in clinical medicine is essential and more in-depth studies involving E. faecalis genes, such as those for regulating biofilm formation, can add important information about the influence of plant extracts on this bacterium. Furthermore, the study contributes to a field of public health importance, which is the introduction of natural extracts in Dentistry, representing a viable alternative as an adjuvant treatment, especially as options with lower toxicity and cost.

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Revista ARACÊ
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