Brazilian Dental Science

unesp
UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
Instituto de Ciência e Tecnologia
Campus de São José dos Campos

ORIGINAL ARTICLE

DOI: https://doi.org/10.4322/bds.2024.e4233

Antifungal effect of *Quillaja saponaria* plant extract on biofilms of five *Candida* species of dental interest

Efeito antifúngico do extrato vegetal de *Quillaja saponaria* sobre biofilmes de cinco espécies de *Candida* de interesse odontológico

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How to cite: Bessa ERL, Muniz AB, Ramos LP, Oliveira LD. Antifungal effect of *Quillaja saponaria* plant extract on biofilms of five *Candida* species of dental interest. Braz Dent Sci. 2024;27(3):e4233. https://doi.org/10.4322/bds.2024.e4233

ABSTRACT

Objective: The objective of this study was to evaluate the action of Q. saponaria glycolic extract on the biofilms of standard strains of *C. albicans*, *C. glabrata*, *C. krusei*, *C. dubliniensis*, and *C. tropicalis*. **Material and Methods:** Monomicrobial biofilms of the five *Candida* species were grown for 48 h, followed by treatment with the isolated extract at five concentrations (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL) and two times of exposure to treatment in all groups (5 min and 24 h), the untreated group, and the group treated with 0.12% chlorhexidine (CLX). To analyze cell viability, the MTT test was used, and the optical densities were transformed into a percentage of metabolic activity. In statistical analysis, data were analyzed by ANOVA and Tukey's test, considering a significance level of 5%. **Results:** The biofilms, when analyzed after a time of 5 minutes, showed fungal reduction when exposed to treatments at 5 concentrations of Quilaia extracts when compared to the untreated group. This applies to the species of *C. albicans*, *C. glabrata*, *C. krusei*, and *C. dubliniensis* ($p<0.0001$), as only the biofilms formed by *C. tropicalis*, despite providing reduction, did not show significant differences between the groups. At 5 minutes, only the biofilms of *C. albicans*, *C. grabrata*, and *C. krusei* treated with Quilaia extract 100 mg/mL showed superior and significant results compared to the group treated with CLX, but at a concentration of 50 mg/mL, only group *C. albicans*. Within 24 h, all groups and all concentrations of Quilaia demonstrated antifungal action ($p<0.0001$). Despite showing a reduction greater than or similar to that promoted by CLX in 24 hours when comparing concentrations of 100 mg/mL and 50 mg/mL, the *C. albicans* groups showed statistically significant differences in this comparison and at this time ($p<0.0001$). **Conclusion:** Therefore, Quilaia extract demonstrated high antifungal potential and was capable of acting on the reduction of *Candida* spp. biofilms at both treatment exposure times and concentrations.

KEYWORDS

Candida; Quilaia; Phytotherapy; Biofilm; Plant extracts.

RESUMO

Objetivo: O objetivo deste estudo foi avaliar a ação do extrato glicólico de Q. saponaria sobre biofilmes de cepas padrão de *C. albicans*, *C. glabrata*, *C. krusei*, *C. dubliniensis* e *C. tropicalis*. **Material e Métodos:** Biofilmes monomicrobianos das cinco espécies de *Candida* foram formados por 48 h, seguido do tratamento com o extrato isolado em cinco concentrações (100 mg/mL, 50 mg/mL, 25 mg/mL, 12,5 mg/mL e 6,25 mg/mL) e em dois tempos de exposição ao tratamento em todos os grupos (5 min e 24 h), grupo sem tratamento e o grupo tratado com clorexidina (CLX) 0,12%. Para análise da viabilidade celular contou com o teste de MTT e as densidades ópticas foram transformadas em percentual de atividade metabólica. Na análise estatística os dados foram analisados por ANOVA e Teste de Tukey, considerando nível de significância de 5%. **Resultados:** Os biofilmes

quando analisados após o tempo de 5 minutos apresentaram redução fúngica quando expostos aos tratamentos nas 5 concentrações de extratos de Quilaia quando comparados com o grupo não tratado, isto se aplica para as espécies de *C. albicans*, *C. glabrata*, *C. krusei* e *C. dubliniensis* (p<0,0001), pois apenas os biofilmes formados por *C. tropicalis*, apesar de proporcionarem redução não apresentaram diferenças significativas entre os grupos. Em 5 minutos, apenas os biofilmes de *C. albicans, C. grabrata* e *C. krusei*, tratados com extrato de Quilaia 100 mg/mL apresentaram resultados superiores e significantes comparados ao grupo tratado com CLX, mas na concentração de 50 mg/mL apenas o grupo *C. albicans*. Em 24 h, todos os grupos e todas as concentrações de Quilaia demonstraram ação antifúngica (p<0,0001). Apesar de apresentarem redução superior ou semelhante a promovida pela CLX em 24 h quando comparadas as concentrações 100 mg/mL e 50 mg/mL os grupos *C. albicans* apresentaram diferenças estatística significantes nesta comparação e neste tempo (p<0,0001). **Conclusão:** Portanto, extrato de Quilaia demonstrou alto potencial antifúngico capaz de atuar na redução de biofilmes de *Candida* spp. em ambos os tempos de exposição ao tratamento e concentrações.

PALAVRAS-CHAVE

Candida; Quilaia*;* Fitoterapia; Biofilme; Extratos de planta.

INTRODUCTION

Candida spp. is a yeast commonly found in the oral cavity of humans; however, as it is an opportunistic fungus, any imbalance in the host-pathogen complex, when the host presents some deficiency or imbalance in the immunological mechanisms, multiplies and penetrates the tissues, causing inflammation, and they stop being colonizers and become pathogens, causing infections, thus causing oral pathologies [1-3].

This genus has several pathogenic species; the most studied and listed in the literature is *Candida albicans*, as it is the predominant species; however, the other species belonging to the non-*albicans Candida* group have demonstrated an increase in prevalence in recent years, leading to a new profile of virulence and pathogenicity and presenting resistance to conventional antifungal treatments [4]. The that are not of the *C. albicans* species are included in a large group of species called non-*albicans Candida*, species that were previously given little emphasis, as they were previously species belonging to infections only in patients with high immunological compromise, but now it has been demonstrated that there is a change in the prevalence profile, where the general population showed an increase in oral infections with these species. This group is composed of: *Candida glabrata*, *Candida tropicalis*, *Candida krusei*, *Candida dubliniensis*, *Candida parapsilosis*, *Candida auris*, among others [5-7].

All these mentioned species count when they become successful pathogens; this makes them successful oral pathogens or less willing to be just human colonizers [8]. The ability of *Candida* spp. to infect various niches of the host is supported by a countless arsenal of virulence factors and attributes of its pathogenic fitness, which is why this genus receives the title of main fungal pathogen [9,10]. Among the main virulence factors that contribute to pathogenicity is the capacity for phenotypic change and the formation of biofilm [11,12].

Due to the fact that the species of *Candida* spp. are involved in high rates of morbidity, new therapeutic approaches have been proposed to achieve antifungal effectiveness, such as the use of herbal medicines, plant extracts, or even chemical compounds extracted from plants [13-15].

Among the plants that have promising components to act as antimicrobials is Quilaia saponária (*Quillaja saponaria*), a plant generally found in western Latin America recognized for having a high content of saponin, a substance with immunoadjuvant properties, as it can stimulate various immune responses [16,17], Quilaia saponária has anti-inflammatory properties and is commonly used for some dermatological diseases [18,19].

Due to the conditions presented, *Candida* spp. has already proven to be a threat to public health as it can cause major impacts on oral and general health, which is why this research was proposed in order to analyze the antifungal effect exerted by Quilaja *saponaria* extract on the biofilms of five ATCC species of *Candida*.

MATERIALS AND METHODS

Glycolic extracts of *Quillaja saponaria* (Quilaia) (Lot: 1475/23280, concentration of 200 mg/mL) obtained from the company Distriol (São Paulo, Brazil) were used. The strains used were reference strains (ATCC, American Type Culture Collection) of *C. albicans* (ATCC 18804), C. *grabrata* (ATCC 9030), *C. krusei* (ATCC 6258), *C. dubliniensis* (ATCC MYA 646), and *C. tropicalis* (ATCC 13803) from the Microbiology and Immunology Laboratory of the Dentistry Course at ICT-UNESP. The broth microdilution method, based on the Clinical and Laboratory Standards Institute (CLSI) M27-S4 standard [20], was applied to determine the minimum inhibitory concentrations (MIC) and minimum fungicidal concentrations (CFM) of the extracts on the fungal strains. *Candida* spp. were cultivated (37°C/24 h) in Sabouraud-dextrose agar (SD-Himedia, Mumbai, India), and then microbial suspensions were prepared where colonies of the respective *Candida* species were diluted in sterile saline solution (NaCl 0.9%) and homogenized in Vortex for 10 s. Standardization was carried out using a spectrophotometer to obtain 10^7 CFU/mL. After preparing the inoculum, standardization was carried out using a spectrophotometer (Micronal B-582, São Paulo, Brazil) using a wavelength of 530 nm and optical density of 0.284.

Subsequently, 200 μL/well of the adjusted *Candida* suspension were added to microplates, which were then incubated at 37°C for 90 min for the initial adhesion of the fungal cells to the well. Then, the supernatant was discarded, and BHI broth (Brain Heart Infusion, Kasvi) was added. Incubation continued for 48 h for biofilm formation, with broth replacement after 24 h of incubation. After the biofilm formation of each species, the supernatant from the wells was discarded, and the corresponding wells were treated with concentrations of *Q. saponaria* extract (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL); the positive control received 0.12% chlorhexidine; the group without treatment; and the control group received only BHI medium. The treatments were carried out in 2 periods: exposure to the extract for 5 min and 24 h.

After exposure to the treatment, the wells were washed with saline solution and discarded to eliminate non-adherent cells that suffered from the action of the therapy. To analyze the biofilms, a viability test of fungal cells was carried out in which 100 μL of MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to each well, and the plates were incubated, protected from light, at 37°C for 1 h. After the incubation period, the MTT solution was removed, followed by the addition of 100 μL of dimethylsulfoxide (DMSO). The plates were again incubated in an oven at 37° C for 10 minutes and placed in the shaker under constant agitation for 10 minutes. Optical densities (OD) were then obtained using a microplate reader at 570 nm, and the OD obtained will be converted into a percentage of the metabolic activity of fungal cells.

Regarding statistical analysis, data that showed normality and homogeneity were analyzed by ANOVA and Tukey's test; otherwise, by the Kruskal-Wallis test and Dunn's test. The GraphPad Prism 5.0 program was used, considering a significance level of 5%.

RESULTS

All biofilms formed by *C. albicans* were evaluated after 5 minutes; one of the groups was not treated, the other received 0.12% chlorhexidine treatment, and the other five groups were treated with 5 concentrations of *Q. saponaria* (100 mg/mL, 50 mg/mL, 25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL) (Figure 1A). The seven groups, when analyzed concomitantly, showed significant statistical differences ($p<0.0001$). The groups treated with *Q. saponaria* extract showed some level of reduction in *C. albicans* biofilms when compared to those that did not receive treatment and those that were treated with 0.12% chlorhexidine, showing a statistically significant difference ($p<0.0001$).

When comparing groups of *C. albicans* biofilms treated with chlorhexidine and the others treated with Quilaia extract (Figure 1B), only the groups that were applied at concentrations of 100 mg/mL and 50 mg/mL showed significant differences ($p <0.0001$), but all showed a decrease in the biofilm product. All groups of *C. albicans* biofilms treated with *Q. saponaria* demonstrated that the higher the concentration of the plant extract, the greater the biofilm reduction ($p = 0.0001$). In the 24-hour treatment time that received *C. albicans* biofilms, the results were similar to the 5-minute treatments, but the groups, when contrasted with each other, showed distinctions.

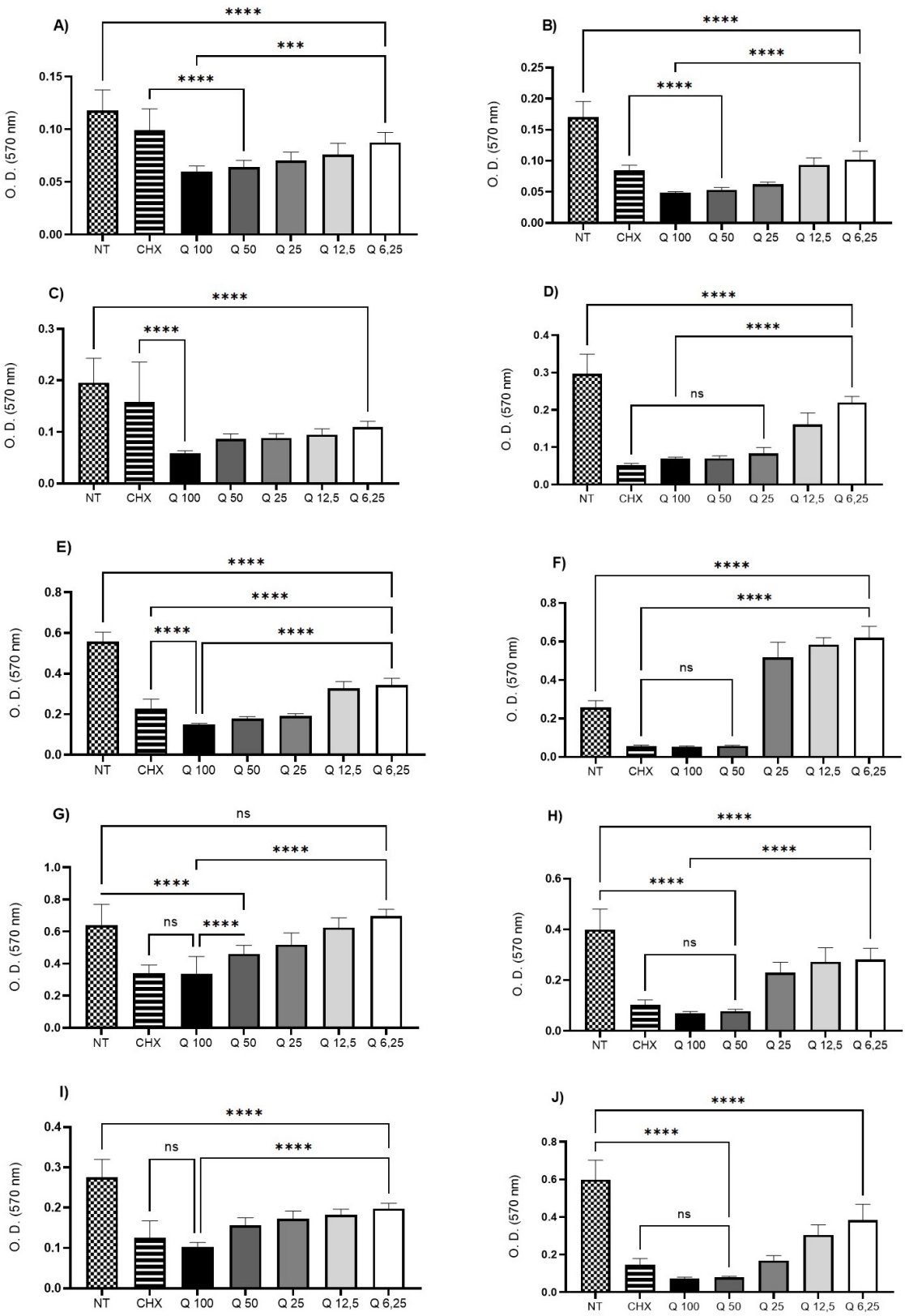

Figure 1. Reduction in the percentage of *C. albicans* biofilm after exposure to treatment with Quilaia stratum for 5 minutes in 24 hours. Captions: NT = Untreated group; CHX = Group treated with 0.12% Chlorhexidine. Q 100 = group that received treatment with 100 mg/mL of Quilaia extract (*Q. saponaria*); Q 50= group that received treatment with 50 mg/mL of Quilaia extract (*Q. saponaria*); Q 25 = group that received treatment with 25 mg/mL of Quilaia extract (*Q. saponaria*); Q 12.5 = group that received treatment with 12.5 mg/mL of Quilaia extract (*Q. saponaria*); Q 6.25 = group that received treatment with 6.25 mg/mL of Quilaia extract (*Q. saponaria*); A) Groups of *C. albicans* biofilms analyzed within 5 minutes; B) Groups of *C. albicans* biofilms analyzed over a 24-hour period; C) Groups of *C. glabrata* biofilms analyzed within 5 minutes; D) Groups of *C. glabrata* biofilms analyzed within 24 hours; E) Groups of *C. krusei* biofilms analyzed within 5 minutes; F) Groups of *C. krusei* biofilms analyzed within 24 hours; G) Groups of *C. tropicalis* biofilms analyzed within 5 minutes; H) Groups of *C. tropicalis* biofilms analyzed over a 24-hour period; I) Groups of *C. dubliniensis* biofilms analyzed within 5 minutes; J) Groups of *C. dubliniensis* biofilms analyzed over a 24-hour period.

When comparing chlorhexidine with the groups treated with extract, all concentrations reduced biofilms, but there were statistical differences in relation to the 100 mg/mL and 50 mg/mL concentrations, which showed the highest levels of reduction ($p<0.0001$), while the concentrations of 12.5 mg/mL and 6.25 mg/mL showed lower reductions than the chlorhexidine group.

Regarding the results of the groups on *C. glabrata* biofilms analyzed after a period of five minutes, as shown in figure 1C, the groups presented different results from each other ($p<0.0001$), the treated groups showed a decrease in the amount of biofilm, and the groups treated with Quilaia extract showed better results when compared to the group treated with chlorhexidine ($p<0.0001$). In the five-minute analysis time (Figure 1D), the twenty-four-hour groups of *C. glabrata* biofilms also showed significant differences in relation to those treated and the group that did not receive treatment ($p<0.0001$).

C. krusei biofilms, at both times analyzed, present significant statistical differences when compared to each other ($p<0.0001$). In figure 1E, composing the five-minute groups, it is observed that the first three analyzed concentrations of Quilaia extract (100 mg/mL, 50 mg/mL, and 25 mg/mL) were more effective in reducing the biofilm of the group treated with chlorhexidine, and the two lowest concentrations (12.5 mg/mL and 6.25 mg/mL) demonstrated lower effectiveness than the treatment, but only the most concentrated extract (100 mg/mL) showed significance in comparison to the group treated with chlorhexidine.

In the analysis of *C. krusei* biofilms over a period of twenty-four hours (Figure 1F), the reduction of the chlorhexidine groups and those treated with Quilaia extract at concentrations of 100 mg/mL and 50 mg/mL showed similar reductions and did not present significant differences; however, the groups treated with the three lowest concentrations (25 mg/mL, 12.5 mg/mL, and 6.25 mg/mL) did not show a reduction in biofilms; on the contrary, they showed an exacerbated increase in *C. krusei* when compared to the other groups ($p<0.0001$). In Figure 1G, we can see the results of the tests analyzed after five minutes on *C. tropicalis* biofilms. The seven groups did not show statistical differences between them, despite the fact that the results of the reduction of biofilms in the groups that were treated with chlorhexidine and with Quilaia extract at 100 mg/mL were similar, but the results were significant when compared to the group that did not receive treatment ($p<0.0001$).

In the groups analyzed after twenty-four hours (Figure 1H), when evaluated together, they showed statistical differences ($p<0.0001$). The two groups of *C. tropicalis* biofilms that were treated with Quilaia extract at 100 mg/mL and 50 mg/mL showed a lower reduction than the group treated with chlorhexidine; despite this, they did not differ statistically; the significant difference was only observed when compared to the group that did not receive treatment.

Figure 1I shows the results of the groups of *C. dubliniensis* biofilms analyzed over a period of 5 minutes. The group with the highest concentration of Quilaia extract (100 mg/mL) was the only one that showed promising results in reducing biofilm when compared with the chlorhexidine group, despite not showing significant differences; however, it showed more than the group that did not receive treatment ($p<0.0001$). In the analysis time of twenty-four hours, we can see in Figure 1J that the two highest concentrations of Quilaia extract showed a reduction in biofilms superior to the other groups; there were significant differences compared to the group that did not receive treatment but not presented in relation to the chlorhexidine group.

DISCUSSION

The present study evaluated the glycolic extract of *Q. saponaria* isolated at different concentrations for treatment against biofilms of *Candida* species: *C. albicans*, *C. glabrata*, *C. tropicalis*, *C. krusei*, and *C. dubliniensis*. It is worth highlighting that most studies involving new therapeutic proposals for this fungal genus, in most cases, choose only *C. albicans* to carry out the analysis [21,22]. However, it is important to highlight that each species of *Candida* has its own characteristics. particularities, which involve everything from its morphology, pathogenesis, and virulence factors, and we can also mention resistance against antifungals [23,24]. This allows us to understand that we cannot treat *Candida* by generalizing as if every infection caused was only caused by species of the *albicans* type, when the literature already allows us to observe studies showing high prevalence rates, virulence, pathogenicity, and resistance of other non-*albicans*

Candida species [25,26]. Therefore, we propose to cover several species to verify the real therapeutic possibility using Quilaia extract. Studies addressing biofilms, this microorganism, and treatment with Quilaia were not found; however, several herbal medicines produced from different plants with anti-*Candida* action are reported in the literature [27]. These studies use the extracts in isolated forms, as in our research, and also evaluate the possibility of a combination with synergistic action with other extracts, such as Mecatti et al. [28] carried out research also analyzing the potential for reducing *Candida* species using glycolic extracts of *Rosa centifolia* L., *Curcuma longa* L., *Rosmarinus officinalis* L., and *Punica granatum* L. against *C. albicans*, *C. dubliniensis*, *C. tropicalis*, and *C. krusei*. This allows us to think of other alternatives for combining Quilaia with other types of plant extracts.

Although we did not find any relationship between *Candida* and Quilaia, other studies presenting the possibility of *Q. saponaria* extracts as an antimicrobial agent were found, addressing other microorganisms such as *Escherichia coli* and *Piscirickettsia salmonis*, but the extracts used were with an aqueous vehicle, different of our study that we used glycolics, however both positive results were independent of the types of extracts used, the antimicrobial action was effective for both bacteria and fungi, as demonstrated by our results, it is important to highlight that, as well as *Candida* species, the two bacteria evaluated also colonize and cause infection in regions of the gastrointestinal and genitourinary tracts, that is, both pathogens have a predilection for the same environment, proving that Quilaia can be a possible and promising therapeutic target for infections in these regions [29,30].

Old records of the use of medicinal plants and the positive results presented by Quilaia were found, both in our results and in some other studies [31]. For decades, Quilaia has been debated as an antimicrobial, as it a has a high content of saponins, which can act on the cell membrane of fungal cells [32,33]. However, there are gaps that need to be filled involving this subject, among which we can mention the cytotoxicity and genotoxicity exerted by this extract. a pharmaceutical form that can be administered to reach superficial and deep tissues [34]. Since *Candida* spp. is a pathogen, it can colonize, cause local and invasive infection, and can reach the bloodstream, which is why the research used two analyzed times: 5 minutes for a drug with a local action and 24 hours to test the extract's ability against biofilms in deep tissues in a longer amount of exposure time, so cellular behavior needs to be analyzed in this case.

The effectiveness of Quilaia, in studies carried out to date, points to a possible medicine for local use. This for dentistry is a highly viable form of drug use, and it would be appropriate to use Quilaia extract as a mouthwash or even as an ointment, as in addition to acting as an antifungal, it would also allow us to stabilize the product, which is one of the other properties of this plant [35]. It is currently being used due to the corresponding saponins in its composition that promote stabilization, so the market uses it in food and beverages, cosmetics handling, photography, and as adjuvants in the production of vaccines [16,36-38]. However, it needs to be verified for the possibility of using Quilaia as an active ingredient and adjuvant for formulation.

Aqueous extracts from the bark of *Q. saponaria* are approved by the United States Food and Drug Administration (FDA) [39] and are also used as a natural flavoring, as they contain bioactive polyphenols, tannins, and triterpenoid saponins with proven anti-inflammatory activity and antimicrobial activity [40]. Saponins, similar to detergents, have cytotoxic, hemolytic, molluscicidal, anti-inflammatory, antifungal, antibacterial, and antiviral activities, and their effects have already been analyzed in prokaryotic and eukaryotic cells [41]. Not only as antimicrobials, but it is scientifically proven that the properties of *Q. saponaria* go beyond infection control; they also achieve preventive benefits for several other diseases, thus acting as a promoter of oral and general health. As a limitation and improvement proposed for the research, there is a need to carry out a photochemical analysis in the future of the extract produced by the supplier company to verify the active ingredients present in this extraction so that we have a complete understanding of the components, since the known properties of Quilaia were provided by literature from other types of extracts and not from laboratory data from our researchers. The need to use clinical strains is also timely considering that new resistance profiles have been reported involving *Candida* spp., so clinical strains with and without a resistance profile are opportune for further studies. One of the species of the genus *Candida*,

C. glabrata, has been highly studied due to the involvement of this microorganism in several deaths, reported in the literature as a successful pathogen, manipulating the immune system, and evading drugs. Our results with the standard strain of this species were promising, which leads us to discuss the need for future trials using clinical and resistant strains, verifying the possibility of Quilaia extract in containing the infection [42-44].

Oral candidiasis presents a new obstacle for oral health, as it has changed its fungal prevalence profile, where previously the highest prevalence was due to species from the *C. albicans* group. However, we observed that many promising studies for the use of new therapeutic therapies still use only the *C. albicans* species in the studies [45], as they still believe that this species is still the main one involved in superficial infections. We can also mention that species that were previously only involved in deep infections are involved in oral infections as well, species of which are not as sensitive as they were in the past to the antifungals that dentists frequently prescribed. Likewise, a new challenge is the formation of polymicrobial biofilms with a much higher resistance profile. Therefore, everything involving this infection currently represents a great challenge, but as it is an oral pathology that promotes an inflammatory response, finding therapeutic protocols capable of combining the anti-inflammatory, antimicrobial, and stabilizing actions of the solution already demonstrates a great achievement for science, about the appropriate control of this pathology.

CONCLUSION

Therefore, Quilaia demonstrated high antifungal potential and was capable of acting on the reduction of biofilms at both times of exposure to treatment, presenting significant statistical differences for all groups, as the higher the concentration, the greater the biofilm reduction. The best results were using Quilaia concentrations at 100 mg/mL and 50 mg/mL, which were superior to the group treated with chlorhexidine for the species of *C. albicans* at both times, *C. glabrata* in five minutes, and *C. krusei* in five minutes.

This study demonstrated that the tested extracts have antifungal potential in *Candida albicans* biofilms, having the ability to influence the decrease in the phenotypic expression of virulence factors, reducing enzymatic secretion, and reaching the proposed objectives which can be indicated as alternative therapeutic tools with the objective of reducing the morbidity of these infections. In both times tested, the secretion of phospholipases, proteases, and hemolyzines produced by *C. albicans* decreased.

Acknowledgements

The authors are grateful to Lucas de Paula Ramos for content contributions and effort to carry out this research, and the Luciane Dias de Oliveira for effective support during the development and writing of these manuscript.

Author's Contributions

ERLB: Review & Editing, Formal Analysis, Investigation, Resources, Data Curation, Writing – Original Draft Preparation. ABM: Review & Editing, Writing – Original Draft Preparation. LPR: Review & Editing, Formal Analysis, Investigation. LDO: Conceptualization, Methodology, Software, Validation, Visualization, Supervision, Project Administration and Funding Acquisition

Conflict of Interest

The authors have no proprietary, financial, or other personal interest of any nature or kind in any product, service, and/or company that is presented in this article.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Regulatory Statement

This study was conducted in accordance with all the provisions of ICT/CSJC – UNESP Ethical Committee Agency. It does not require approval from the ethics committee because the research does not involve humans or animals.

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Date submitted: 2024 Jan 17
Accept submission: 2024 Aug 21