Comparative Immunology, Microbiology and Infectious Diseases 65 (2019) 226–233

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Control of bacterial and fungal biofilms by natural products of *Ziziphus joazeiro* Mart. (Rhamnaceae)

Jacqueline Cosmo Andrade[a,b], Ana Raquel Pereira da Silva[a], Maria Audilene Freitas[c], Bárbara de Azevedo Ramos[d], Thiago Sampaio Freitas[a], Franz de Assis G. dos Santos[c], Melyna C. Leite-Andrade[c], Michellângelo Nunes[c], Saulo Relison Tintino[a], Márcia Vanusa da Silva[d], Maria Tereza dos Santos Correia[d], Reginaldo Gonçalves de Lima-Neto[e], Rejane P. Neves[c], Henrique Douglas Melo Coutinho[a,*]

[a] *Laboratory of Microbiology and Molecular Biology – LMBM, University Regional of Cariri – URCA, Crato, CE Brazil*
[b] *Laboratory Bioassay – LABIO, University Federal of Cariri – UFCA, Institute of Educators Training, Campus Brejo Santo, CE, Brazil*
[c] *Laboratory of Medical Mycology Sylvio Campos, University Federal of Pernambuco – UFPE, Recife, PE, Brazil*
[d] *Center of Biological Sciences and Department of Biochemistry, University Federal of Pernambuco – UFPE, Recife, PE, Brazil*
[e] *Department of Tropical Medicine, University Federal of Pernambuco – UFPE, Recife, PE, Brazil*

ARTICLE INFO

Keywords:
Microbial biofilms
Stem bark
Ethnoknowledge
Leaf
Chlorhexidine gluconate
Saponins

ABSTRACT

The aim of this study is to verify the action of the aqueous leaf extract *Ziziphus joazeiro* in the eradication of bacterial and fungal biofilms, and to compare these effects with the stem bark extracts, as well as with conventional standard drugs. The presence of secondary metabolites was observed through phytochemical prospection assays. The effect of the aqueous extract on microbial biofilm formation was observed by OD600 nm absorbance and the crystal violet assay. For bacterial and fungal biofilms, chlorhexidine gluconate and fluconazole, respectively, were used as positive controls. Phytochemical characterization showed the presence of secondary metabolite classes common to both extracts such as flavonoids, steroids and saponins. In particular, in the aqueous leaf extract phenols, condensed tannins and alkaloids were observed. Eradication results using the aqueous leaf extract showed an inhibition of the microbial biofilm mass, moreover the biofilms were more sensitive to the bark extract, which presented a greater inhibition number and an action similar to standard drugs. It is important to highlight the leaf extract showed significant eradication at the lowest concentrations for mature yeast biofilms, thus demonstrating its potential to modify microbial resistance susceptibility. Bacterial and fungal biofilm eradication results using the *Ziziphus joazeiro* aqueous extracts presented a biofilm inhibition effect for both, moreover the results support the ethnopharmacological knowledge surrounding the use of *Ziziphus joazeiro* stems in the community. In comparison, the bark extract presented a more effective treatment than the leaf extract against biofilms, presenting inhibition levels similar to the used standard drugs.

1. Introduction

Biofilms are microbial ecosystem complexes characterized by one or more microbial cell community, covered by an extracellular polymeric structure which adheres to a biotic and/or abiotic subtract [1,2].

This complex represents one of the main microbial resistance mechanisms, due to evolutionary advantages this confers to it, especially protection against extreme conditions, such as lack of nutrients, pH and temperature changes, free radicals and ultraviolet radiation, in addition to the action of antibiotics and even the action of the host's immune system [3].

Biofilm development is a serious healthcare concern given persistent infections, comprising roughly 80% or more of microbial infectious cases [4,5].

Bacterial infections caused by biofilm proliferation such as chronic, nosocomial and medical device associated infections, have an increased resistance to conventional antibiotics when compared to isolated infections [6]. Among the bacterial resistance mechanisms, biofilms induce physiological changes in response to scarcity, stimulate efflux pump expression, participate in *quorum sensing* and provides an environment for genetic interaction and transfer, thus contributing to the dissemination of tolerance mechanisms against various drugs of clinical interest [7].

* Corresponding author at: Street Cel. Antônio Luís 1161, Pimenta, 63105-000, Brazil.
E-mail addresses: hdmcoutinho@gmail.com, hdmcoutinho@urca.br (H.D. Melo Coutinho).

https://doi.org/10.1016/j.cimid.2019.06.006
Received 20 March 2019; Received in revised form 11 June 2019; Accepted 13 June 2019

Infections caused by the *Candida* genus represent the fourth greatest cause of infections associated with healthcare [8]. *Candida* spp. pathogenicity is directly linked to its virulence factors, such as dimorphism, extracellular enzyme production, their ability to adhere to surfaces and their ease in forming biofilms. Biofilm formation is the main clinical repercussion, especially due to the increased resistance of certain species to antifungal therapy [9,10].

Therefore, biofilms represent a source of infection and are associated with a growing mortality index when compared to the same species isolate which does not form a biofilm [11].

In this context, the implementation of natural products with a therapeutic focus, which are already used by the population, occurs due to the high antimicrobial resistance incidence. Plant extracts are being increasingly studied and considered as possible sources of new compounds with antimicrobial activity [12].

According to ethnobiological studies, *Ziziphus joazeiro* Mart. is an endemic plant from the Caatinga, commonly used as an oral antiseptic [13], antimycotic [14], expectorant, as well as in the treatment of bronchitis and gastric ulcers [15].

Ziziphus joazeiro Mart. is commonly used by the population in oral hygiene and gingivitis treatment. Plant parts, especially the bark and stem bark, are triturated and used for tooth brushing. From conservative point of view, the incorrect practice of stem removal may lead to girdling, a process of total or partial removal of the bark in the circumference of the plant, which impedes nutrient and water flow, resulting in tissue dehydration, in addition to leaving the plant exposed to pest and microorganismal attacks causing plant death [16]. In turn, the leaves are used for the manufacture of anti-dandruff shampoo and capillary tonic, as well as for washing utensils [17,18].

Buccal cavities possess a vast and diverse microbial flora, composed by bacteria and fungi, with *Streptococcus mutans*, *Staphylococcus epidermidis* and yeasts such as *Candida* spp. being commonly found. These coexist, interact and grow in biofilms, which can range from a simple dental plaque to other oral diseases, such as periodontal disease, endodontic infections, angular cheilitis and prosthetic stomatitis [19].

Ziziphus joazeiro bark and leaves have in their phytochemical constitution, saponins, which in aqueous solutions form persistent and abundant foam, this being responsible for the property that makes the *Ziziphus joazeiro* stem bark be used by the population for dental brushing [18].

Due to the *Ziziphus joazeiro* traditional medicine application and the continuous removal of its stem bark by the population, it is necessary to validate the use of another *Z. joazeiro* part in order to minimize stem bark usage. Therefore, the aim of this study is to verify the action of the aqueous leaf extract against microbial resistance in the eradication of bacterial and fungal biofilms, and to compare this with the aqueous stem bark extract and chlorhexidine gluconate effects, a chemical already used in oral hygiene, as well as fluconazole.

2. Materials and methods

2.1. Collection area and plant material

Ziziphus joareizo Mart. leaves and stem barks were collected from Sítio Ipueiras, located in the rural area of the Brejo Santo municipality, South of Ceará, Brazil, at the foot of the Chapada do Araripe, with geographic coordinates 07°28’54.4”S/39°01’47.2”W. Collections took place in February 2017. A species sample was deposited in the Dárdano de Andrade Lima Herbarium of the Regional University of Cariri – URCA under n°13.346 and identified as *Ziziphus joazeiro* Mart. (Rhamnaceae).

2.2. Extract preparation

Z. joazeiro leaf and stem bark aqueous extracts (EAFZJ and EACCZJ) were produced by cold extraction maceration [20]. Fresh leaves were cut and the stem barks were dried at room temperature and crushed in a mechanical mill. Posteriorly, both were added to distilled sterile water, packaged in a container protected from light and air, and after 72 h, the extracts were filtered, frozen and taken to a lyophilizer (−60 °C).

2.3. Qualitative chemical prospection

Chemical assays qualitatively analysed the presence of secondary metabolites. The method based on Matos [21] evaluated the presence of phenols, tannins, flavonoids, alkaloids, steroids. To verify steroids and/or triterpenoids, the Liebermann – Burchard test was performed using an adapted method from Campbell and Shawn [22].

2.4. Biofilm production evaluation and bacterial strain eradication

2.4.1. Used strains

Six bacterial lineages were used: *Streptococcus mutans* INCQS 00446 (ATCC 25175), *Enterococcus faecalis* INCQS 00018 (ATCC 14506) and *Staphylococcus epidermidis* INCQS 00016 (ATCC 12228), obtained from the Microorganism Collection Reference in Sanitary Surveillance (CMRVS), Oswaldo Cruz Foundation - FIOCRUZ - INCQS, Rio de Janeiro, RJ; *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 9027 and *Escherichia coli* ATCC 259223, obtained from the Mycology Laboratory of the Federal University of Paraíba (UFPB). To perform the assays, each sample was subcultured in a BHI agar environment and incubated at 37 °C for 24 h, after which a small amount of cells were removed and diluted in 0.9% NaCl and adjusted in a spectrophotometer (600 nm) to a concentration of 5×10^5 CFU/mL (5×10^4 CFU/μL well).

2.4.2. Solution preparations for biofilm assays

Product solutions were prepared through a 20 mg and 2 mg dilution for each extract, with concentrations ranging from 2 mg/mL to 0.2 mg/mL. 0,12% Chlorhexidine gluconate (Kley) was used as the standard reference antibiofilm.

2.4.3. Biofilm formation induction

Induction was performed according to the methodology by Trentin et al. [23] with some modifications. For biofilm production, 96-well microdilution plates were used. 20 μL of 0.85% NaCl, 20 μL of the bacterial inoculum and 160 μL of the brain-heart infusion (BHI) broth medium were added to each well and were incubated for 24 h at 37 °C. After incubation, the contents were removed and the dishes were washed three times with sterile 0.85% saline with the biofilms being fixed by incubating at 55 °C for 1 h.

2.4.4. Biofilm formation evaluation

Biofilm formation using isolates were evaluated by the Violet Crystal method described by Stepanovic et al. [24] in different culture medium and formation surfaces using microtiter plates. Through optical density (OD) readings, average absorbance values were determined for each sample (ODa), compared to the absorbance of the sterility control (ODc). Samples were classified as strongly (4x ODc < ODa), moderately (2x ODc < ODa < 4x ODc) and weakly (ODc < ODa < 2x ODc) biofilm forming agents. Isolates presenting absorbance values equal to or lower than the control were classified as non-biofilm producers.

2.4.5. Biofilm treatment

After biofilm preparation, 20 μL of the different extract concentrations and of chlorhexidine gluconate were added to the microtiter plate with 20 μL of the bacteria and 160 μL of the brain-heart infusion broth (BHI) growing media. The plate was incubated for 24 h at 37 °C, where the contents were removed after incubation and the plate was washed three times with 0.85% saline solution, incubated at 55 °C for 1 h, and colored with 0.4% CV for 15 min. This was subsequently washed three times with saline solution and eluted in ethanol (100%). The reading was performed with an absorbance of 570 nm.

2.4.6. Statistical analysis

Statistical analysis were performed using the software *Graphpad Prism*, v. 5.0. Data were analyzed using the arithmetic mean of the absorbances obtained from each extract for each bacterium in up to 8 replicates. Data were subsequently analyzed using a one-way ANOVA ($P < 0.05$; *$P < 0.1$; ****$P < 0.0001$), followed by Tukey's post-hoc test. Each variable was compared to the biofilm growth control and chlorhexidine gluconate.

2.5. Biofilm production evaluation and Candida genus eradication

2.5.1. Used strains

Standard lineages were obtained from the Oswaldo Cruz Culture Collection (FIOCRUZ) of the Brazilian Institute of Quality Control in Health (INCQS). *Candida albicans* INCQS 40006, *Candida tropicalis* INCQS 40042 and isolated strains were obtained from the Culture collection of the Federal University of Pernambuco - URM (Recife Mycology University) *Candida albicans* URM 4387, *Candida tropicalis* URM 4262. The strains were inoculated in Sabouraud Dextrose Agar (SDA, KASVI) and incubated for 24 h at 37 °C. An initial suspension was subsequently prepared in 5 mL of sterile saline solution (NaCl, 0.85% saline) and its density was adjusted according to the 0.5 MacFarland scale with 90% transmittance determined by spectrophotometry, using a wavelength of 530 nm. This provides a standard yeast concentration containing from 1×10^6 to 5×10^6 cells per mL.

2.5.2. Preparation of solutions for biofilm assays

Products solutions were prepared through dilution of 0.15 g of each extract followed by further dilution in 1 mL of dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany). The tested concentrations varied from 16.384 μg/mL to 128 μg/mL. Fluconazole (Pfizer) was used as reference antifungal drug with concentrations varying from 64 μg/mL to 0.5 μg/mL.

2.5.3. Qualitative evaluation of Candida spp. biofilm development

Qualitative evaluation of biofilm formation capacity was performed using the visual method adapted from Shin et al. [25]. After seeding the isolates in Sabouraud agar medium and preparing the suspensions as described above, 20 μL were inoculated into 180 μL of liquid Sabouraud contained in the microplate wells, which were maintained at 35 °C for 24 h and 48 h without shaking. The contents were then aspirated and the wells were washed with distilled sterile water and a Fuchsin dye (QEEL-Specialized Chemistry Erich Ltda.) was added to perform the evaluation according to the intensity of the staining. Staining was evaluated visually and classified as follows: 1) Strongly stained: when the biofilm was intensely stained allowing the correct determination of the contour of the areas containing the biofilm; 2) Average staining: when the biofilm was stained more weakly but still allowed the determination of the contour of the areas containing the biofilm; 3) Poor staining: when the biofilm was not stained making it impossible to distinguish the color of the plate and the characteristic colour of the stain. The interpretation represented a strong, moderate and weak biofilm formation activity, respectively.

2.5.4. Biofilm formation induction

96-well microdilution plates were used for biofilm production where, 20 μL of the suspensions were transferred to microplate wells containing 180 μL of YPD (*Yeast extract-Peptone-Dextrose*). The plates were incubated at 37 °C for 48 h. Following the incubation time, the wells were carefully aspirated and washed twice with 200 μL of PBS buffer.

The prewashed wells were stained with 110 μL of 0.4% aqueous violet crystal solution for 45 min. Thereafter, they were washed three times with 200 μL of ultrapure sterile water (Milli-Q) and the biofilm was discoloured using 200 μL ethanol for 45 min. At the end, 100 μL from each well were transferred to a new microplate and biofilm formation was evaluated using the optical density difference between the biofilm formed and the control well, by reading the absorbance in a spectrophotometer adjusted with a wavelength of 595 nm. Each strain was tested three times and the absorbance values of the control well were subtracted from the tested wells to minimize interference [26].

2.5.5. Biofilm treatment

Biofilms were formed onto microdilution plates, as aforementioned. After a 48 h period, wells with biofilms were filled with 200 μL of the eight *Ziziphus joazeiro* (EAFZJ and EACCZJ) aqueous extracts and Fluconazole serial dilutions. The extracts and standard drug were diluted in RPMI 1640 to achieve each concentration. Untreated biofilm wells and biofilm free wells were included as positive and negative controls respectively. The microdilution plates were incubated for 24 h and 48 h at 35 °C. After each period, the biofilm was quantified as previously described [27].

2.5.6. Statistical analysis

For statistical analysis, the *Graphpad Prism* v. 5.0. software was used. Data were analyzed using the arithmetical mean from triplicates for each tested concentration and posteriorly analysed using a two-way ANOVA ($P < 0.05$; *$P < 0.1$; ****$P < 0.0001$), comparing values for each extract concentration, point by point, using Bonferroni's post-hoc test. A general behaviour comparison for each substance against the tested strains was inferred through the already cited analysis.

3. Results

Aqueous extract production from the stem and leaves presented a yield of 9.51% and 8.54%, respectively.

EAFZJ and EACCZJ phytochemical characterization saw the presence of secondary metabolite classes common to both extracts such as flavonoids (leucoanthocyanidins, flavones, flavonols, flavononols, flavonones, xanthones, catechins), steroids and saponins. In particular, phenols, condensed tannins and alkaloids were observed in the EAFZJ.

Table 1 and Fig. 1 show the biofilm formation capacity of the tested bacterial strains, including *Escherichia coli* which presented minimal biofilm formation, being eliminated during treatment assays.

In the *Streptococcus mutans* biofilm eradication (Fig. 2), the extracts presented a lower biofilm growth percentage than the growth control at all concentrations used. Treatment with EAFZJ and EACCZJ at a concentration of 0.2 mg/mL inhibited biofilm mass by 46.4% and 48.5%, respectively, presenting a similar action than chlorhexidine gluconate, with an inhibition of 46.8%.

The 0.2 mg/mL EAFZJ concentration was the only one which presented biomass reduction compared to the growth control against *Enterococcus faecalis* (Fig. 3) biofilms; however, its inhibition was not significant, when compared to chlorhexidine.

As for *Staphylococcus* biofilm treatment (Fig. 4), a significant inhibitory extract effect was observed against *Staphylococcus epidermidis* biofilms, when compared to the growth control, with 0.2 mg/mL EAFZJ and EACCZJ concentrations inhibiting biofilm mass by 40% and 36.5%,

Table 1
Evaluation of bacterial biofilm formation by optical density (570 nm).

Clinical isolates	Biofilm formation
Streptococcus mutans INCQS 00446 (*ATCC* 25175)	Moderate
Enterococcus faecalis INCQS 00018 (*ATCC* 14506)	Moderate
Staphylococcus epidermidis INCQS 00016 (*ATCC* 12228)	Strong
Pseudomonas aeruginosa ATCC 9027	Moderate
Escherichia coli ATCC 259223	weak

INCQS - Brazilian Institute of Quality Control in Health.

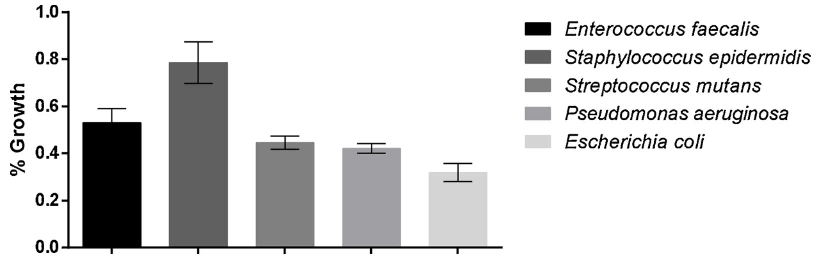

Fig. 1. Capacity of biofilm formation by bacterial strains by (%) growth.

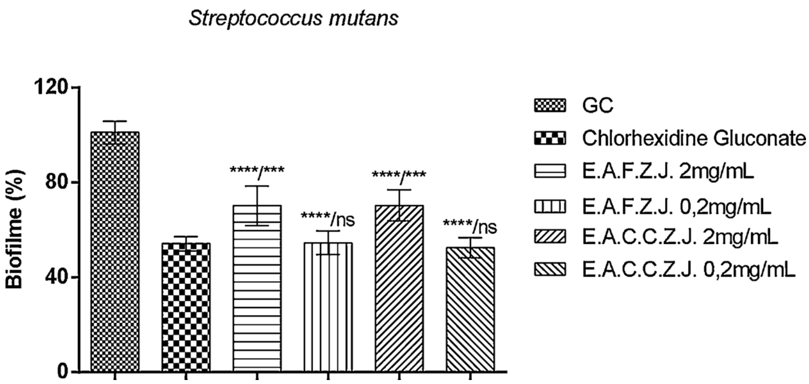

Fig. 2. Eradication of *Streptococcus mutans* biofilm by aqueous extracts of Ziziphus joazeiro, compared to 12% chlorhexidine gluconate. On the columns are the statistical relevance of the substance in relation: growth control/chlorhexidine gluconate. $p < 0.05$; * ($p > 0.01$); **** ($p < 0.0001$); ns = not significant. GC. Growth control. E.A.F.Z.J. Aqueous extract of the leaves of *Ziziphus joazeiro*. E.A.C.C.Z.J. Aqueous extract of the bark of the stems of *Ziziphus joazeiro*.

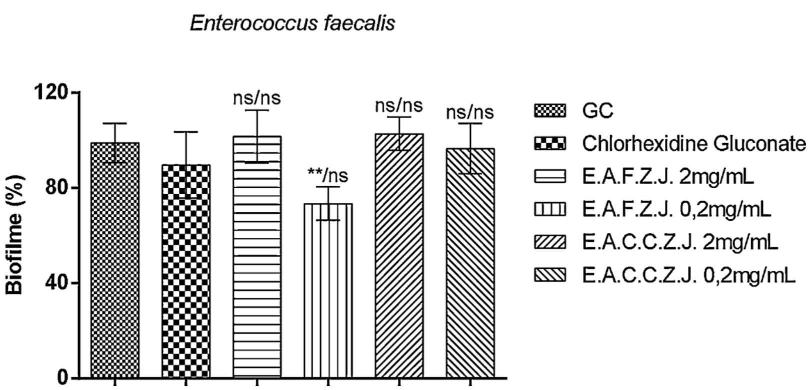

Fig. 3. Eradication of *Enterococcus faecalis* biofilm by aqueous extracts of Ziziphus joazeiro, compared to 12% chlorhexidine gluconate. On the columns are the statistical relevance of the substance in relation: growth control / chlorhexidine gluconate. $p < 0.05$; * ($p > 0.01$); **** ($p < 0.0001$); ns = not significant. GC. Growth control. E.A.F.Z.J. Aqueous extract of the leaves of *Ziziphus joazeiro*. E.A.C.C.Z.J. Aqueous extract of the bark of the stems of *Ziziphus joazeiro*.

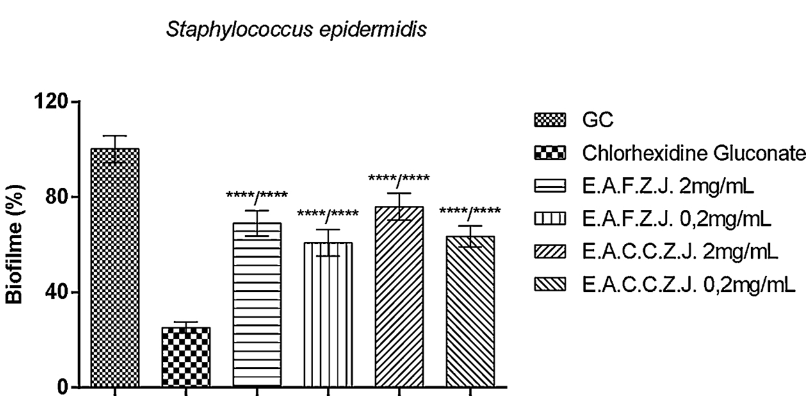

Fig. 4. Eradication of *Staphylococcus epidermidis* biofilm by aqueous extracts of Ziziphus joazeiro, compared to 12% chlorhexidine gluconate. On the columns are the statistical relevance of the substance in relation: growth control / chlorhexidine gluconate. $p < 0.05$; * ($p > 0.01$); **** ($p < 0.0001$); ns = not significant. GC. Growth control. E.A.F.Z.J. Aqueous extract of the leaves of *Ziziphus joazeiro*. E.A.C.C.Z.J. Aqueous extract of the bark of the stems of *Ziziphus joazeiro*.

respectively. However, these did not present a significant result when compared to the standard reference drug, chlorhexidine gluconate, which inhibited 74.5% of biofilm formation.

Results from the extracts' influence on *Pseudomonas aeruginosa* biofilm formation are shown in Fig. 5. All extract concentrations used in the treatment presented significant results against the positive control, with 2 mg/mL EAFZJ and EACCZJ concentrations inhibiting biofilm formation by 45.8% and 45.3%, and the 0.2 mg/mL concentrations inhibition biofilm formation by 46.4% and 73%. EACCZJ biofilm mass inhibition approached the inhibition of chlorhexidine gluconate, which showed a reduction of 80%.

The EAFZJ eradication results demonstrate bacterial biofilm mass inhibition, however, biofilms were more susceptible to the EACCZJ extract, which presented higher inhibition numbers and an action

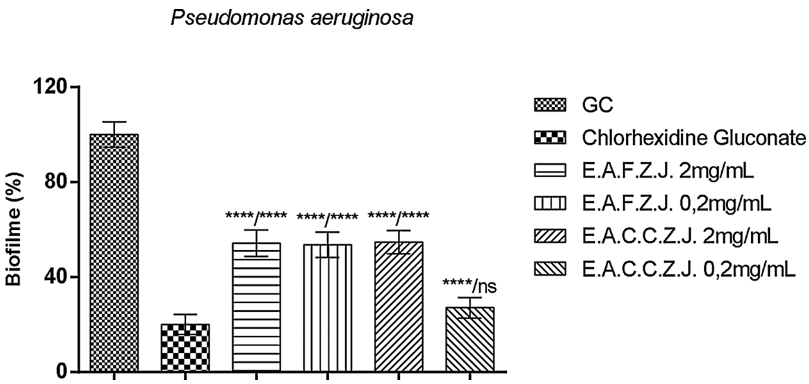

Fig. 5. Eradication of *Pseudomonas aeruginosa* biofilm by aqueous extracts of Ziziphus joazeiro, compared to 12% chlorhexidine gluconate. On the columns are the statistical relevance of the substance in relation: growth control/chlorhexidine gluconate. $p < 0.05$; * ($p > 0.01$); **** ($p < 0.0001$); ns = not significant. GC. Growth control. E.A.F.Z.J. Aqueous extract of the leaves of *Ziziphus joazeiro*. E.A.C.C.Z.J. Aqueous extract of the bark of the stems of *Ziziphus joazeiro*.

Table 2
Evaluation of biofilm formation by violet crystal method.

Clinical isolates	Biofilm formation
Candida albicans INCQS 40006	Strong
Candida albicans URM 4387	Moderate
Candida tropicalis INCQS 40042	Strong
Candida tropicalis URM 4262	Strong

INCQS - Brazilian Institute of Quality Control in Health.; URM - University Recife Mycology.

similar to that of chlorhexidine gluconate against the tested strains.

The biofilm formation capacity of clinical *Candida* spp. isolates are shown in Table 2, with the interpretation being performed taking into account colour intensity.

Fig. 6 presents the biofilm induction results obtained based on optical density values, with all yeast isolates being capable of forming biofilms within 24 and 48 h, with different intensities. *C. tropicalis* isolates presented, when statistically compared to others, a higher capacity for biofilm production URM 4262 (2.494 nm) and INCQS 40042 (2.487 nm), followed by *C. albicans* INCQS 40006 (1.780 nm) and URM 4387 (1.873 nm).

Treatment of biofilms formed by *C. albicans* (Fig. 7) isolates demonstrate significant inhibition (58.8%) by the EACZJ against the INCQS 40006 strain, 24 h, at the lowest concentration, when compared to the EAFZJ (14.6%). At 48 h, inhibition using both EAFZJ concentrations is noticeable, with the effect at the highest concentration standing out, obtaining an inhibition value of 94.4% (59.9% for the EACZJ; 72.2% for fluconazole). For URM 4387 isolates, inhibition occurs only in the 24 h biofilm formation assays, using a concentration of 128 μg/mL; the EACZJ presented a percentage inhibition of 61.1%, being statistically the best result when compared to the EAFZJ (8.7%) and fluconazole (10.6%). At the 16.384 μg/mL concentration, an inversion of the results was observed with the EAFZJ standing out against the EACZJ, obtaining an 88.4% inhibition, a result similar to the standard drug (99.1%).

Fig. 8 shows the *C. tropicalis* biofilm formation results for the INCQS 40042 strain, 24 h, with biofilm reduction occurring at the lowest and highest EACZJ concentrations, inhibiting biofilm formation by 59.1% and 74.4%, respectively. Thus presenting significant inhibition when compared to EAFZJ (7.9% and 38.9%) and the conventional antifungal (25.5% and 69.3%). In the 48 h assay, the lowest EAFZJ concentration induced an inhibition of 75.5%, standing out from the EACZJ (35.1%) and fluconazole (25.5%). Treatment with the URM 4262 isolate was statistically relevant at 24 h with the 128 μg/mL concentration, where the EACZJ reduced biofilm biomass by approximately 90.5%.

With the results in mind, the EAFZJ presented a greater biofilm formation inhibition in the 48 h assays, that is, in mature biofilms, whereas the EACZF acted in intermediate biofilms formed within 24 h. In addition, the EACZJ inhibitory actions over biofilms formed by *Candida* isolates, occurred in greater numbers when compared to the EAFZJ.

4. Discussion

Phytochemical analysis using *Ziziphus joazeiro* leaves, bark and stem bark extracts report the presence of flavonoids, alkaloids, steroids, tannins, saponins, carbohydrates and cellulose [28–31].

Several phenolic compounds such as flavonoids, tannins and saponins, present antimicrobial activity and act as fungal and bacterial biofilm formation inhibitors and biomass inhibitors [32,33].

The probable secondary metabolite antimicrobial and antibiofilm mechanisms of action are listed as cytoplasmic membrane trauma, effective inhibition of cell metabolism enzymes and microbial aggregation inhibition [34].

Bacterial biofilm formation capacity is determined by the particulars of each species and by a variety of adhesion site factors [35]. Albano et al. [5] cite *Staphylococcus epidermidis, Staphylococcus aureus* and *Pseudomonas aeruginosa* as species with a high proliferation capacity in medical devices and biofilm development, within clinically relevant biofilm formation bacterial strains.

Candida spp. biofilm formation is species-dependent, this meaning, biofilm formation is conditioned to the species type and environmental conditions, where *Candida albicans* and non-*albicans* strains create biofilms in a similar way, though with well-defined characteristics [36]. *Candida albicans* and *Candida tropicalis* are the *Candida*'s species which most form biofilms [37].

C. albicans isolates possess thicker and less compact biofilms, with the presence of yeasts in basal parts and filamentous forms in superior parts [10]. *C. tropicalis* biofilms are an agglomerate of cells and filaments, with these differences contributing to the pathogenic potential of each lineage [38].

The biofilm formation process in *Candida* spp. is separated into three steps: an initial, intermediary and a mature phase. In the mature phase, specifically within 48 h, the entire biofilm is covered by the polymeric extracellular matrix and displays a three-dimensional growth, where all resistance mechanisms are established and active in this phase [39].

With respect to biofilm inhibition, the results support the ethnopharmacological data highlighting the use of the *Ziziphus joazeiro* stem barks by the community as toothpaste [17]. In contrast, studies carried out with *Ziziphus joazeiro* aqueous bark extracts [40] and leaf essential oils [41] were not active against bacterial and fungal biofilms.

Some studies cite the antimicrobial activity of *Ziziphus joazeiro* [14–43], however, it is important to note that to reduce biofilm biomass, inhibitory concentrations of up to ten times greater than the usual concentrations needed to inhibit planktonic cells [44] are necessary.

In view of the bacterial biofilm eradication results, the 0.2 mg/mL concentration presented the highest biofilm formation inhibition, when compared to the 2 mg/mL concentration, probably due to a higher

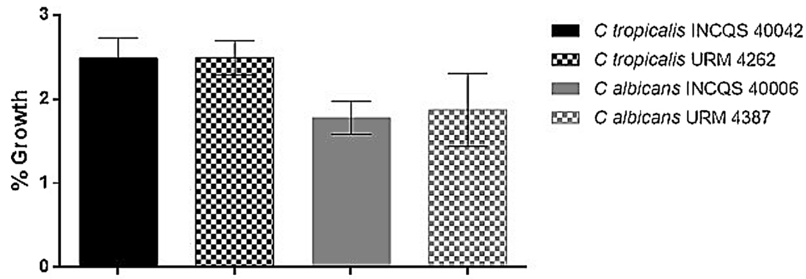

Fig. 6. Biofilm formation of clinical isolates of *Candida* spp.

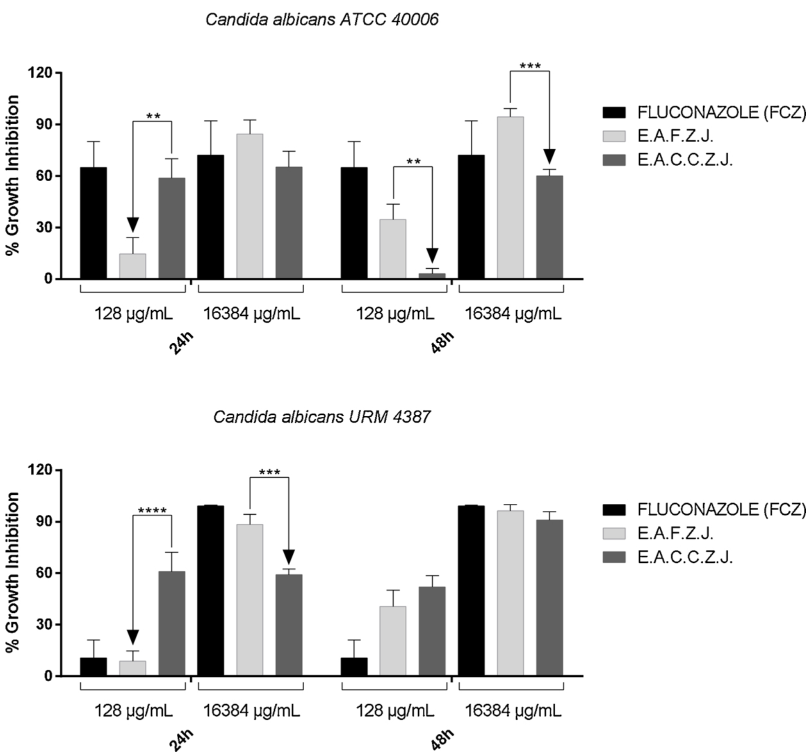

Fig. 7. Inhibition of biofilm formation (%) of isolates *Candida albicans*, in 24 and 48 h, by treatment of the aqueous extracts of Ziziphus joazeiro Mart. Compared with the antifungal standard fluconazole. (* $P < 0.1$; **** $P < 0.0001$). E.A.F.Z.J. Aqueous extract of the leaves of *Ziziphus joazeiro*. E.A.C.C.Z.J. Aqueous extract of the bark of the stems of *Ziziphus joazeiro*.

nutrient subsidy present in the higher extract concentrations, such as proteins and polysaccharides, which instead of reducing, contribute to biofilm development and formation [3].

Among the extracts, the EACCZJ presented greater *Streptococcus mutans* and *Pseudomonas aeruginosa* inhibition, inhibiting similarly or superiorly, when compared to chlorhexidine gluconate. Chlorhexidine is an antimicrobial agent with a broad spectrum against gram-positive and gram-negative bacteria, yeast, dermatophytes and some viruses [45].

Chlorhexidine gluconate is one of the most popular antiseptics used in the prevention and inhibition of caries and gingivitis at concentrations ranging between 0.12% and 2%. Its action against biofilms consists of microbial lysis, due to cellular permeability modifications or coagulation of bacterial cytoplasmic compounds, in addition to reducing microbial adhesion to contact surfaces [46,47].

The significant EACCZ treatment results against some biofilm strains may be associated with its chemical composition, especially secondary metabolites, such as saponins. These have antimicrobial, insecticide and molluscicide properties, in addition to interacting with bacterial membrane lipids and consequently increasing cellular permeability through channel formation and/or membrane rupture [42–48].

Microscopy and biochemical studies performed by Parai et al. [49] indicate *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilm eradication may be associated with the loss of extracellular polymer substances present in the bacterial biofilm matrix and alterations in cell membrane integrity of the bacterial strains by saponins. The action of saponins in mature *Candida albicans* biofilm eradication is reported by Sadowska et al., who highlight a cellular wall instability and reduced yeast adherence during biofilm formation.

When comparing isolated yeast biofilm formation periods, almost no biofilm biomass inhibition was noticed in the 48 h period with the highest concentration (16.384 µg/mL). According to Gutierrez et al. [50], a lower biomass reduction and/or biofilm increase when exposed to an extract, is a specificity of yeast species, which produce marked filamentation in an attempt to defend and enhance its virulence, to overcome stress and death by the presence of these natural products.

The phytochemical extract composition is another factor, which may contribute nutrients for extracellular matrix development, thus guaranteeing greater biofilm resistance. The matrix from *Candida* species consists mainly of carbohydrates, proteins, phosphate and hexosamines; the *Ziziphus joazeiro* aqueous extract possesses a considerable amount of carbohydrates [18].

A study performed by Paula-Mattiello et al. [51], using *C. parapsilosis* biofilms formed between 6, 12 and 24 h showed cell viability loss when these were treated with ketoconazole, while biofilms from

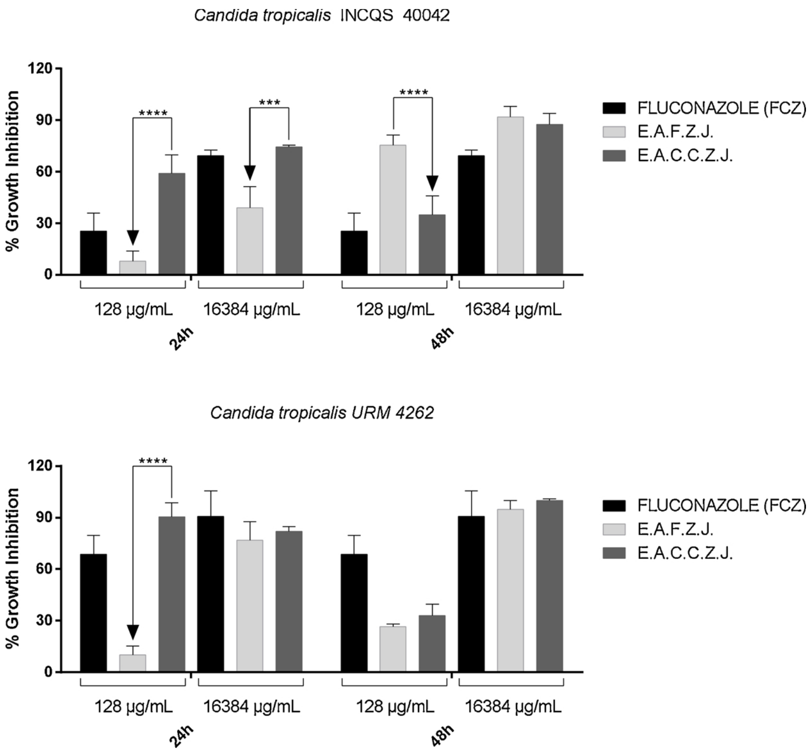

Fig. 8. Inhibition of biofilm formation (%) of isolates *Candida tropicalis*, in 24 and 48 h, by treatment of the aqueous extracts of Ziziphus joazeiro Mart. Compared with the antifungal standard fluconazole. (* P < 0.1; **** P < 0.0001). E.A.F.Z.J. Aqueous extract of the leaves of *Ziziphus joazeiro*. E.A.C.C.Z.J. Aqueous extract of the bark of the stems of *Ziziphus joazeiro*.

the same species were resistant to this antifungals after a 48 h period, thus showing a greater structural complexity of the mature biofilm.

The treatment of mature biofilms by standard drugs and natural substances are mostly ineffective, which is probably due to the complexity and resistance of their structure, such as the extracellular matrix, which acts as a barrier neutralizing substances and the presence of resistant cells at high concentrations, the *persister*, which collaborate with the resistance and insistence of fungal biofilm infections [52].

Thus, it is important to highlight the EAFZJ result, which showed a significant inhibition against *Candida* biofilms formed within 48 h, this reduction being relevant given it shows a change in mature biofilm susceptibility and resistance. The phytochemical composition of the leaf extract revealed the presence of condensed tannins which, in mature *Candida* sp. biofilms, can interact with polysaccharides, inactivating enzymes responsible for maintaining the extracellular matrix, thus reducing biofilm mass and cell surface hydrophobicity [53].

5. Conclusion

Eradication of fungal and bacterial biofilms using the *Ziziphus joazeiro* aqueous extracts indicate a biofilm inhibition effect for both fungi and bacteria. Moreover, results support the ethnopharmacological knowledge regarding *Ziziphus joazeiro* stem bark usage by community. The EACCZJ presented more effective results against biofilms than the EAFZJ, obtaining inhibition values similar to standard drugs. Additionally, the EAFZJ presented significant eradication of mature yeast biofilms, at the lowest concentrations, thus demonstrating its potential in modifying microbial resistance susceptibility. Lastly, further studies addressing the mechanisms of action of the aforementioned extracts against biofilms are necessary.

Acknowledgements

We thank to Laboratory of Medical Mycology Sylvio Campos and the Center of Biological Sciences and Department of Biochemistry, University Federal of Pernambuco, for their partnership in biofilm assays.

References

[1] P. Messi, Biofilm formation, development and relevance, Biofilm in Bioengineering, Nova Science, Hauppauge, New York, 2013.
[2] C. Condò, I. Anacarso, C. Sabia, R. Iseppi, I. Anfelli, et al., Antimicrobial activity of spices essential oils and its effectiveness on mature biofilms of human pathogens, Nat. Prod. Res. 13 (2018) 1–8.
[3] N. Cerca, N.F. Azevedo, Biofilmes na saúde, no meio ambiente, na indústria, Publindústria, Braga, Porto, 2012.
[4] H. Pelling, J. Nzakizwanayo, S. Milo, E.L. Denham, W.M. MacFarlane, et al., Bacterial biofilm formation on indwelling urethral catheters, Lett. Appl. Microbiol. 68 (2019) 277–293.
[5] M. Albano, B.P. Crulhas, F.C.B. Alves, A.F.M. Pereira, B.F.M.T. Andrade, et al., Antibacterial and anti-biofilm activities of cinnamaldehyde against S. epidermidis, Microb. Pathog. 126 (2019) 231–238.
[6] Z. Khatoon, C.D. McTiernan, E.J. Suuronen, T.F. Mah, E.I. Alarcon, Bacterial biofilm formation on implantable devices and approaches to its treatment and prevention, Heliyon 12 (2018) 10–67.
[7] T. Jiang, M. Li, Quorum sensing inhibitors: a patent review, Expert Opin. Ther. Pat. 7 (2013) 867–894.
[8] D.P. Santana, T.F. Ribeiro, E.L. Ribeiro, G.L.B. Aquino, P.L.F. Naves, Ação de chalconas contra a formação de biofilme de *Candida albicans*, Rev Ciênc Farm Básica Apl. 36 (2015) 83–90.
[9] G. Ramage, J.P. Martínez, J.L. López-Ribot, *Candida* biofilms on implanted biomaterials: a clinically significant problem, FEMS Yeast Res. 6 (2006) 979–986.
[10] D.P. Santana, E.L. Ribeiro, A.C.S. Menezes, P.L. Faleiro, Novas abordagens sobre os fatores de virulência de *Candida albicans*, Rev Ciênc Méd Biol. 12 (2013) 229–233.
[11] F. Nagy, Z. Tóth, A. Bozó, A. Czeglédi, Rebenku István, et al., Fluconazole is not inferior than caspofungin, micafungin or amphotericin B in the presence of 50% human serum against *Candida albicans* and *Candida parapsilosis* biofilms, Med. Mycol. 0 (2018) 1–9.
[12] E.A.M. Bona, F.G.S. Pinto, T.K. Fruet, T.C.M. Jorge, A.C. Moura, Comparação de métodos para avaliação da atividade antimicrobiana e determinação da concentração inibitória mínima (cim) de extratos vegetais aquosos e etanólicos, Arq. Inst. Biol. 81 (2014) 218–225.
[13] H. Lorenzi, F.J.A. Matos, Plantas medicinais no Brasil:nativas e exóticas, Instituto Plantarum, Nova Odessa – São Paulo, 2008.
[14] M.C.S. Cruz, P.O. Santos, A.M. Barbosa Jr., D.L.F.M. Melo, C.S. Alviano, et al., Antifungal activity of Brazilian medicinal plants involved in popular treatment of mycoses, J. Ethnopharmacol. 111 (2007) 409–412.
[15] H.E. Lorenzi, Árvores brasileiras:manual de identificação e cultivo de plantas arbóreas nativas do Brasil, Instituto Plantarum, Nova Odessa – São Paulo, 2009.
[16] M.O. Santos, Efeito do Anelamento do caule sobre as relações fonte-dreno em plantas de *Inga vera* WILLD, Dissertação – Pós-graduação em Fisiologia Vegetal. (2009).
[17] M.T. Cavalcanti, D.C. Silveira, I.M. Florêncio, V.A. Feitosa, Eller SCWS, Obtenção da farinha do fruto do juazeiro (*Ziziphus joazeiro* Mart.) e Teor de caracterização físico-química, Revista Verde de Agroecologia e Desenvolvimento Sustentável. 6 (2011)

220–224.
[18] F.C.P. Dantas, M.L.R. Tavares, M.S. Targino, A.P. Costa, F.O. Dantas, *Ziziphus joazeiro* Mart. - Rhamnaceae: características biogeoquímicas e importância no bioma Caatinga, RES Principal 25 (2014) 51–57.
[19] L. Dutreix, C. Bernard, C. Juin, C. Imbert, M. Girardot, Do raspberry extracts and fractions have antifungal or anti-adherent potential against *Candida* spp.? Int. J. Antimicrob. Agents 52 (2018) 947–953.
[20] F.J.A. Matos, Introdução à fitoquímica experimental, terceira ed., Editora UFC, Fortaleza, Ceará, 2009.
[21] F.J.A. Matos, Farmácias vivas, 4 ed., Editora UFC, Fortaleza, 2002, pp. 36–40.
[22] M.K. Campbell, O.F. Shawn, Biochemistry, 4th ed., Thomson Asia pte Ltd, Singapore, 2005.
[23] D.S. Trentin, R.B. Giordani, K.R. Zimmer, A.G. Silva, M.V. Silva, et al., Potential of medicinal plants from the Brazilian semi-arid region (Caatinga) against Staphylococcus epidermidis planktonic and biofilm lifestyles, J. Ethnopharmacol. 137 (2011) 327–335.
[24] S. Stepanovic, D. Vukovic, I. Dakic, B. Savic, M. Svabic-Vlahovic, A modified microtiter-plate test for quantification of staphylococcal biofilm formation, J. Microbiol. Methods 40 (2000) 175–179.
[25] J.H. Shin, S.J. Kee, M.G. Shin, S.H. Kim, D.H. Shin, et al., Biofilm production by isolates of *Candida* species recovered from nonneutropenic patients: comparison of bloodstream isolates with isolates from other sources, J. Clin. Microbiol. 40 (2002) 1244–1248.
[26] A.S. Melo, F.C. Bizerra, E. Freymuller, B.A. Arthington-Skaggs, A.L. Colombo, Biofilm production and evaluation of antifungal susceptibility amongst clinical *Candida* spp. isolates, including strains of the *Candida parapsilosis* complex, Med. Mycol. 49 (2011) 253–262.
[27] M. Prazinska, E. Gospodarek, In vitro effect of amphotericin B *on Candida albicans, Candida glabrata* and *Candida parapsilosis* biofilm formation, Mycopathologia 177 (2002) 19–27.
[28] E. Fumagali, R.A.C. Gonçalves, M.F.P.S. Machado, G.J. Vidoti, A.J.B. Oliveira, Produção de metabólitos secundários em cultura de células e tecidos de plantas: o exemplo dos gêneros Tabernaemontana e Aspidosperma, Rev bras farmacogn. 18 (2008) 627–641.
[29] T.C.D.L. Silva, C.C.B.R. Almeida, J. Veras Filho, T. Peixoto Sobrinho, E.L.C. Amorin, et al., Atividades antioxidante e antimicrobiana de Ziziphus joazeiro Mart. (Rhamnaceae): avaliação comparativa entre cascas e folhas, Rev Ciênc Farm Básica Apl. 32 (2011) 193–199.
[30] M.S.F. Melo, C.Q. Rocha, M.H. Santos, J.M. Chavasco, J.K. Chavasco, Pesquisa de bioativos com atividade antimicrobiana nos extratos hidroetanólicos do fruto, folha e casca de caule do Ziziphus joazeiro Mart, Rev Universidade do Vale Verde 10 (2012) 43–51.
[31] S.M. Brito, H.D. Coutinho, A. Talvani, C. Coronel, A.G. Barbosa, et al., Analysis of bioactivities and chemical composition of *Ziziphus joazeiro* Mart. using HPLC-DAD, Food Chem. 1 (2015) 185–191.
[32] M. Xing, F. Shen, L. Liu, Z. Chen, N. Guo, et al., Antimicrobial efficacy of the alkaloid harmaline alone and in combination with chlorhexidine digluconate against clinical isolates of *Staphylococcus aureus* grown in planktonic and biofilm cultures, Lett. Appl. Microbiol. 54 (2012) 475–482.
[33] A. Borges, C. Ferreira, M.J. Saavedra, M. Simões, Antibacterial activity and mode of action of ferulic and gallic acids against pathogenic bacteria, Microb. Drug Resist. 19 (2013) 256–265.
[34] T.P. Cushnie, A.J. Lamb, Recent advances in understanding the antibacterial properties of flavonoids, Int. J. Antimicrob. Agent 38 (2011) 99–107.
[35] T.J. Foster, J.A. Geoghegan, V.K. Ganesh, M. HOOk, Adhesion, invasion and evasion: the many functions of the surface proteins of *Staphylococcus aureus*, Nat. Rev. Microbiol. 12 (2014) 49–62.
[36] A. Henriques, M. Negri, S. Silva, O impacto clínico de biofilmes de espécies de *Candida*, Biofilmes na saúde, no meio ambiente, na indústria, Publindústria, Braga, Porto, 2012.
[37] A. Mohammed, L. Al-Fattani, J. Douglas, Biofilm matrix of *Candida albicans* and *Candida tropicalis*: chemical composition and role in drug resistance, J. Med. Microbiol. 5 (2006) 999–1008.
[38] M. Negri, S. Silva, M. Henriques, J. Azeredo, T. Svidzinski, et al., *Candida tropicalis* biofilms: artificial urine, urinary catheters and flow model, Med. Mycol. 49 (2011) 739–747.
[39] M. Ghannoum, E. Roilides, A. Katragkou, V. Petraitis, T.J. Walsh, The role of echinocandins in *Candida* biofilm-related vascular catheter infections: in vitro and in vivo model systems, Clin. Infect. Dis. 6 (2015) 618–621.
[40] W.S. Alviano, D.S. Alviano, C.G. Diniz, A.R. Antoniolli, C. Alviano, et al., *In vitro* antioxidant potential of medicinal plant extracts and their activities against oral bacteria based on Brazilian folk medicine, Arch. Oral Biol. 53 (2008) 545–552.
[41] S.M. Bersan, L.C. Galvão, V.F. Goes, A. Sartoratto, G.M. Figueira, et al., Action of essential oils from Brazilian native and exotic medicinal species on oral biofilms, BMC Complement. Altern. Med. 18 (2014) 441–451.
[42] B.D. Ribeiro, D.S. Alviano, D.W. Barreto, M.A.Z. Coelho, Functional properties of saponins from sisal (*Agave sisalana*) and juá (*Ziziphus joazeiro*): critical micellar concentration, antioxidant andantimicrobial activities, Colloids Surf. A: Physicochem. Eng. Aspects 436 (2013) 736–743.
[43] L. Scorzoni, F. Sangalli-Leite, S.J. Lacorte, S.A.C. Paula, C.B. Costa-Orlandi, et al., Searching new antifungals: the use of in vitro and in vivo methods for evaluation of natural compounds, J. Microbiol. Methods 123 (2016) 68–78.
[44] J. Bueno, Anti-biofilm drug susceptibility testing methods: looking for new strategies against resistance mechanism, J. Microb. Biochem. Technol. 3 (2014) 1–9.
[45] Y. Sakaue, S. Takenaka, T. Ohsumi, H. Domon, Y. Terao, et al., The effect of chlorhexidine on dental calculus formation: an in vitro study, BMC Oral Health 18 (2018) 1–7.
[46] S. Kumar, S. Patel, J. Tadakamadla, H. Tibdewal, P. Duraiswamy, et al., Effectiveness of a mouthrinse containing active ingredients in addition to chlorhexidine and triclosan compared with chlorhexidine and triclosan rinses on plaque, gingivitis, supragingival calculus and extrinsic staining, Int. J. Dent. Hyg. 11 (2013) 35–40.
[47] D. Kohli, S.M. Hugar, K.G. Bhat, P.P. Shah, M.V. Mundada, et al., Comparative evaluation of the antimicrobial susceptibility and cytotoxicity of husk extract of *Cocos nucifera* and chlorhexidine as irrigating solutions against *Enterococcus faecalis*, *Prevotella intermedia* and *Porphyromonas gingivalis*—an *in-vitro* study, J. Indian Soc. Pedod. Prev. Dent. 36 (2018) 142–150.
[48] M.I. Khan, A. Abdulatef, H.S. Jin, S.B. Jun, Y.K. Min, et al., Green tea seed isolated saponins exerts antibacterial effects against various strains of gram positive and gram negative bacteria, a comprehensive study in vitro and in vivo, J. Evid. Complement. Altern. Med. 12 (2018) 1–12.
[49] D. Parai, E. Islam, J. Mitra, S.K. Mukherjee, Effect of Bacoside A on growth and biofilm formation by Staphylococcus aurous and Pseudomonas aeruginosa, Can. J. Microbiol. 63 (2017) 169–178.
[50] J.K. Trigo-Gutierrez, P.V. Sanitá, A.C. Tedesco, A.C. Pavarina, E.G.O. Mima, Effect of Chloroaluminium phthalocyanine in cationic nanoemulsion on photoinactivation of multispecies biofilm, Photodiagn. Photodyn. Ther. 24 (2018) 212–219.
[51] S. Paula-Mattiello, S.D. Oliveira, R. Medina-Silva, In vitro evaluation of hydrolytic enzyme activity and biofilm formation of *Candida parapsilosis* species complex from a nosocomial environment, Rev. Soc. Bras. Med. Trop. 50 (2017) 558–561.
[52] J. Wuyts, P. Van Dijck, M. Holtappels, Fungal persister cells: the basis for recalcitrant infections? PLoS Pathog. 8 (2018) 1–14.
[53] A.T. Morey, F.C. Souza, J.P. Santos, C.A. Pereira, J.D. Cardoso, et al., Antifungal activity of condensed tannins from *Stryphnodendron adstringens*: effect on *Candida tropicalis* growth and adhesion properties, Curr. Pharm. Biotechnol. 17 (2016) 365–375.