



Matricaria Recutita Extract (Chamomile) to reduce Candida Albicans and Enterobacter Cloacae biofilms: in vitro study

Extrato de Matricaria recutita (camomila) para redução do biofilme de Candida albicans e Enterobacter cloacae: estudo in vitro


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ABSTRACT

Objective

This research study aimed at evaluating the inhibitory activity of Matricaria recutita (chamomile) hydroalcoholic extract on Candida albicans and Enterobacter cloacae biofilms.

Methods

C. albicans and E. cloacae biofilms with thirty-hour formation were submitted, for five minutes, to 100, 200 and 300 mg / mL of M. recutita hydroalcoholic extract, chlorhexidine digluconate 0.12% (Periogard® - inhibition control) or sterile distilled water (growth control). Subsequently, they were washed and divided into two groups to determine the microbial viability: GI/UC - counting of colony forming units (cfu) in agar and GI/DNA - quantification of viable DNA with violet crystal dye by spectrophotometry.

Results

M. recutita extract at 300 mg/mL reduced significantly ($p < 0.01$) the E. cloacae cfu/mL number in biofilm with results similar to chlorhexidine 0.12%, while extracts at 100 and 200 mg/mL did not have the same effectiveness. The amount of E. cloacae viable DNA was reduced ($p < 0.05$) in all the M. recutita extract concentrations and chlorhexidine. There was no significant difference ($p = 0.565$) in the cfu/mL number or in the amount of viable DNA ($p = 0.8094$) in C. albicans biofilm when compared to untreated biofilm (control) or, even, between the extracts when compared to each other or to chlorhexidine 0.12%.

Conclusion

300 mg/mL M. recutita extract reduced significantly the E. cloacae biofilm but not the C. albicans, both with a similar result to chlorhexidine 0.12% (Periogard®).

Indexing terms: Biofilms. Candida Albicans. Enterobacter Cloacae. Matricaria. Phytotherapy.

RESUMO

Objetivo

Avaliar a atividade inibitória do extrato hidroalcoólico de Matricaria recutita (camomila) sobre biofilme de Candida albicans e Enterobacter cloacae.

Métodos

Biofilmes de C. albicans e E. cloacae com trinta horas de formação foram submetidos por cinco minutos a 100, 200 e 300 mg/mL de extrato hidroalcoólico de M. recutita, digluconato de clorexidina 0,12% (Periogard® - controle de inibição) ou água destilada esterilizada (controle do crescimento). Depois foram lavados e divididos em dois grupos para determinação da viabilidade microbiana: G1 - contagem de unidades formadoras de colônia (ufc) em ágar e G2 - quantificação de DNA viável com corante cristal violeta por espectrofotometria.

Resultados

O extrato de M. recutita a 300 mg/mL reduziu significativamente ($p < 0,01$) o número de ufc/mL de E. cloacae em biofilme com resultados semelhantes a clorexidina 0,12%, enquanto os extratos a 100 e 200 mg/mL não tiveram a mesma efetividade. Já a quantidade de DNA viável de E. cloacae foi reduzida ($p < 0,05$) em todas as concentrações do extrato de M. recutita testadas e clorexidina. Não houve diferença significativa ($p=0,565$) no número de ufc/mL ou na quantidade de DNA viável ($p=0,8094$) no biofilme de C. albicans quando comparado ao biofilme sem tratamento (controle) ou mesmo entre as concentrações do extrato quando comparados entre si ou com a clorexidina 0,12%.

Conclusão

O extrato de M. recutita 300 mg/mL reduziu significativamente o biofilme de E. cloacae mas não de C. albicans, ambos com resultado semelhante à clorexidina 0,12% (Periogard®).

Termos de indexação: Biofilme. Candida albicans. Enterobacter cloacae. Matricaria. Fitoterapia.

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INTRODUCTION

Among the microorganisms capable of biofilm formation, causing oral diseases in predisposing conditions, are the yeasts of the genus *Candida*, mainly the *C. albicans* and the enteric gram-negative bacilli such as the *E. cloacae*.

C. albicans is a member of the normal human microbiota and colonizes the oral cavity, the gastrointestinal tract and the genitourinary tract of 70%, or more, of the population [1], as a commensal agent, but, inducing infection. In the mouth, it occurs when there is an alteration in such environment or immunological dysfunction conditions [2]. The ability of this species to transition from a commensal to a pathogenic agent is related to a repertoire of virulence factors, such as adhesion capacity to the mucosa, epithelium and artificial materials, including medical and dental devices (catheters, probes, pacemakers etc.), dimorphism with the production of filamentous structures causing tissue invasion, significant thermo-tolerance and production of enzymes such as proteinases and phospholipases [3-5]. This enzyme, located on the surface of the yeast and at the end of the germ tube, acts on the phospholipids hydrolysis and causes damage to the epithelial cell [6].

The *Enterobacter* is a gram-negative common genus, facultative anaerobic, bat-shaped bacteria belonging to the *Enterobacteriaceae* family. The *E. cloacae* species has clinical significance as an opportunistic bacteria and is a hospital pathogen in intensive care patients [7]. Resistance to β -lactam and extended-spectrum β -lactam (ESBL) often complicates the treatment of *E. cloacae* related infections [8]. This species is not considered to be part of the oral cavity, however, it was the most commonly isolated species among the facultative anaerobic gram-negative microorganisms in adult subjects, presenting chronic periodontitis [9] and in tongue backs of men and women between 30-50 years of age [10].

In addition to the mechanical control, the biofilm chemical control is recommended as well; however, some substances can generate side effects such as tooth staining, taste alterations, soft tissue burns, pain, xerostomia, scaly lesions, ulcerations and unpleasant taste in the mouth [11].

As an alternative for biofilm control, studies conducted with herbal medicine have demonstrated its applicability in clinical practice, mainly to treat and prevent less severe conditions [12]. The use of this

resource to treat the population has been one of the goals of the Brazilian Ministry of Health, especially to treat those whose socioeconomic conditions are unfavorable to buy manufactured drugs. This initiative is based on the National Policy on Integrative and Complementary Practices at SUS, approved by the National Health Council in 2005 and published by Decree GM No. 971, dated May 3, 2006, proposing the inclusion of medicinal plants and phytotherapy as a therapeutic option in the public health system [13].

In Dentistry, researches with natural products have increased in recent years, because besides presenting a more affordable cost to the population, their acceptance leads to good prospects in dental market products containing natural substances [14].

Matricaria recutita also known as chamomile can be an alternative for use in Dentistry. Plants from the *Asteraceae* family, from European origin, grows in mild climates, but can adapt to warmer climates. It has an intense and sweet aroma, presenting anxiolytic, digestive and sedative properties, but, in Dentistry, the properties that stand out are its antiseptic and anti-inflammatory properties [15].

Therefore, the goal of this research study was to evaluate the inhibitory activity of the *M. recutita* hydroalcoholic extract on *C. albicans* and *E. cloacae* biofilms to contribute to the use of this phytotherapeutic agent in Dentistry.

METHODS

Considering the in vitro tests, *C. albicans* (ATCC 18804) and *E. cloacae* (CCUT 01003) strains from the cultures collection of the University of Taubaté were used in this research study.

The minimum inhibitory concentration (MIC) of the *M. recutita* hydroalcoholic extract (Farmaway, Taubaté, São Paulo, Brazil) on strains in the planktonic phase was determined by the broth microdilution method [16]. In order to verify the extract action on the biofilm, the MIC was used for the planktonic phase and higher concentrations, until reaching the extract maximum concentration (300 mg / mL).

For the biofilm formation, each strain was seeded in brain heart infusion broth (BHI, Kasvi, Roseto degli Abruzzi, Italy) and incubated at 37°C for 24 hours, centrifuged (Centribio TDL80-2B centrifuge, Shanghai,

China) at 3700 rpm for ten minutes and the supernatant discarded. The deposit was resuspended in sterile saline (NaCl 0.9%), centrifuged and the supernatant discarded. This procedure was repeated three times for waste removal from the culture medium. From the reservoir, *C. albicans* and *E. cloacae* suspensions were prepared in sterile saline solution, adjusted at 106 and 108 cels/mL, respectively, in a spectrophotometer (Femto 432C, São Paulo, Brazil - wavelength: 530 and 590nm , respectively).

In 96 well microdilution plates (SPL Life Sciences, Gyeonggi-do, Korea), 200 µL of the *C. albicans* or *E. cloacae* suspension were added, incubated at 37°C under agitation (75 rpm, Biomixer TS multifunctional stirrer -2000, China) for 90 minutes for initial adhesion. Then, the culture medium was removed and each well was washed three times with sterile saline to remove unbound cells. 200 µL of BHI broth was added to the washed wells, incubated at 37°C for 24 hours and, then, the culture medium was removed and each well washed three times with sterile saline to remove unbound cells.

Twelve repetitions were performed for each group (Table 1) at two different times, totaling 24 repetitions for each group (Chart 1).

Table 1. Optical densities for *Candida albicans* cell viability test in untreated (control) biofilm and treated with *Matricaria recutita* extract (83, 100, 200 and 300 mg / mL) and chlorhexidine 0.12%.

<i>Matricaria recutita</i> extract (mg/ mL)				Chlorhexidine 0.12%	Control
83	100	200	300		
0.142	0.128	0.145	0.151	0.130	0.148
0.142	0.092	0.124	0.13	0.177	0.139
0.085	0.104	0.148	0.123	0.077	0.294
0.142	0.215	0.193	0.179	0.143	0.104
0.083	0.152	0.162	0.142	0.246	0.129
0.065	0.097	0.104	0.118	0.092	0.093
0.054	0.050	0.100	0.105	0.059	0.096
0.067	0.064	0.114	0.104	0.057	0.097
0.123	0.076	0.071	0.155	0.077	0.094
0.182	0.093	0.062	0.170	0.139	0.151
0.143	0.137	0.117	0.107	0.107	0.143
0.156	0.140	0.113	0.144	0.189	0.078

Anova, gl = 5, p=0.8094

Chart 1. Microorganisms, groups and treatments used in biofilm assays, wherein: (*) Sodium Chloride (NaCl) 0.9% and (**) Periogard®, Colgate-Palmolive Company, Brazil.

Microorganism	Gr	Treatment
<i>Candida albicans</i>	1	Sterilized saline solution (positive control) *
	2	Chlorhexidine 0.12% (negative control) **
	3	<i>M. recutita</i> extract 83 mg/mL (MIC)
	4	<i>M. recutita</i> extract 100 mg/mL
	5	<i>M. recutita</i> extract 200 mg/mL
	6	<i>M. recutita</i> extract 300 mg/mL
<i>Enterobacter cloacae</i>	7	Sterilized saline solution (positive control) *
	8	Chlorhexidine 0.12% (negative control) **
	9	<i>M. recutita</i> extract 100 mg/mL (MIC)
	10	<i>M. recutita</i> extract 200 mg/mL
	11	<i>M. recutita</i> extract 300 mg/mL

For the biofilm treatment, 200 µL of *M. recutita* hydroalcoholic extract were added to the wells with different concentrations or control solutions (sterile saline solution or chlorhexidine 0.12%), as shown in Table 1, where they remained for five minutes. Then, they were removed and the biofilm washed three times with sterile saline, as described above, for extract removal or control solution.

After washing, twelve wells from each group received 200 µL of sterile saline solution and the biofilm cells were detached by friction with a sterile tip. From the detached cell suspension, multiple serial dilution of two (10⁻² to 10⁻⁸) and plated (G / CFU) were performed by the drop technique [17] on BHI agar (Kasvi, Roseto degli Abruzzi, Italy). The media were incubated for 24 hours at 37°C and the reading performed by counting and calculation of the colony forming units per milliliter (cfu/ml). The cfu/ml numbers were transformed into base 10 logarithms.

For the cell viability test (G/DNA), 100 µL of formaldehyde 10% (Chemco, Hortolândia, São Paulo, Brazil) were added to each one of the twelve remaining wells in each group, being removed after ten minutes (fixation). The wells were washed with 200 µL of sterile saline and then 100 µL of violet crystal dye were added (Dynamics, Brazil, 0.2 mg/mL sterile distilled water concentration). The plate was covered with foil and incubated at 37°C under agitation for 15 minutes (75 rpm - TS-2000A Multifunctional Shaker). After discarding the supernatant, each well was washed with 200µL of distilled water and then 100µL of 95% ethyl alcohol were added (Synth, Labsynth, Diadema, São Paulo, Brazil). The reading

was performed on a microplate spectrophotometer (Versamax, Molecular Devices, USA) using ethyl alcohol as white (λ 530 and 590 nm) [5].

The cfu / mL log data and optical densities were analyzed applying the one-way ANOVA (Graphpad Prism 5.0) test, considering a significance level of 5%.

RESULTS

The minimum inhibitory concentration of the *M. recutita* extract for *C. albicans* and *E. cloacae* was 83 and 100 mg / mL in the planktonic phase, respectively. Both were inhibited by chlorhexidine 0.12% (Periogard® 0.12%).

Considering the biofilm study, there was a percentage reduction in the number of cfu/mL of *C. albicans* for all concentrations of *M. recutita* extract and chlorhexidine 0.12% (Md 72.4 to 99.6%). However, when comparing the efficacy between the concentrations, although there was a decrease in the log of cfu / mL with the increase in extract concentration, this difference was not significant, even when compared to chlorhexidine 0.12% ($p = 0.565$, Figure 1).

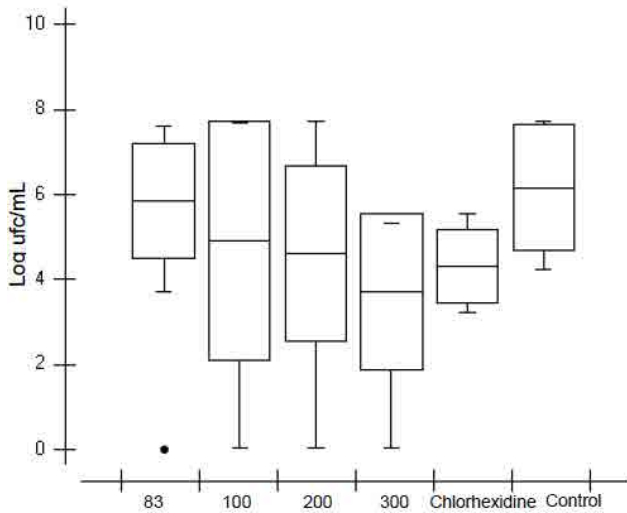


Figure 1. Mean and standard deviation of cfu log/ml of untreated *Candida albicans* biofilm (control) or treated for five minutes with *Matricaria recutita* extract at 83, 100, 200 and 300 mg/ml and chlorhexidine 0.12% (ANOVA, $p = 0.565$).

When comparing the optical densities obtained in the cell viability test in *C. albicans* biofilm, there was no significant difference ($p = 0.8094$) in the amount of viable DNA between the control and the tested concentrations of *M. recutita* extract (83,100, 200, 300 mg / mL) and chlorhexidine 0.12% (Table 1).

For the *E. cloacae* biofilm, the *M. recutita* extract at 300 mg/mL significantly reduced ($p < 0.01$) the number of cfu/mL, when compared to the control, with results similar to chlorhexidine 0.12%, while the extracts at 100 and 200 mg/mL did not have the same effectiveness (Figure 2).

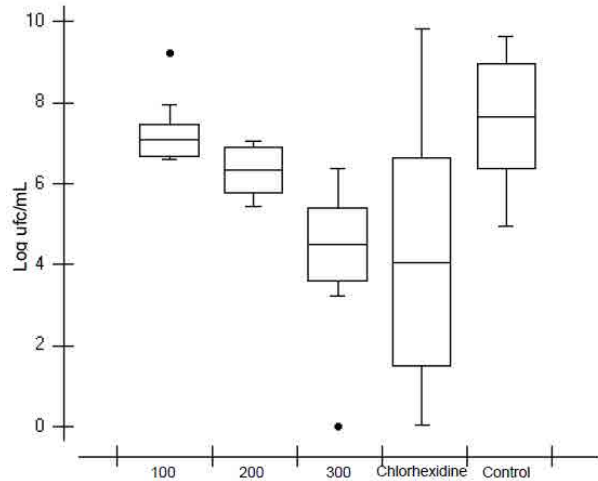


Figure 2. Mean and standard deviation of log cfu/ml of untreated *Enterobacter cloacae* biofilm (control) or treated for five minutes with *Matricaria recutita* extract at 100, 200 and 300 mg / ml and chlorhexidine 0.12% (ANOVA, $p < 0.01$).

For the *E. cloacae* cell viability test, the analysis of optical densities showed that *M. recutita* extract in all the tested concentrations (100, 200 and 300 mg/mL), as well as chlorhexidine 12%, significantly reduced the biofilm viable DNA ($p < 0.05$) when compared to the control. There was no difference between the extract concentrations and between each concentration and the chlorhexidine 0.12% in the reduction of viable DNA (Table 2).

Table 2. Optical densities for the cell viability test of *Enterobacter cloacae* in untreated biofilm (control) and treated with *Matricaria recutita* extract (100, 200 and 300 mg / mL) and chlorhexidine 0.12%.

<i>Matricaria recutita</i> Extract (mg/mL)			Chlorhexidine 0.12%	Control
10%	20%	30%		
0.098	0.117	0.121	0.746	1.207
0.11	0.14	0.095	0.135	0.69
0.336	0.177	0.118	0.168	0.479
0.12	0.213	0.139	0.159	0.921
0.163	0.325	0.119	0.336	0.419
0.122	0.088	0.325	0.189	0.221
0.064	0.071	0.285	0.166	0.053
0.062	0.066	0.078	0.042	0.055
0.127	0.078	0.056	0.035	0.145
0.066	0.049	0.055	0.093	0.159
0.072	0.085	0.101	0.061	0.101
0.061	0.182	0.105	0.034	0.121

ANOVA, $p < 0.05$

DISCUSSION

The most common and effective strategy to prevent caries, gingivitis, periodontitis and other oral conditions related to biofilm accumulation is its mechanical removal from dental surfaces, restorations or dental prostheses by regular brushing [18]. However, it is often necessary to use mouthwashes with antimicrobial substances to assist in the biofilm control.

The search for a drug capable of reducing microorganisms, which can be pathogenic in various situations in the oral cavity, not causing side effects, especially when indicated for prolonged use, is appropriate and assists in obtaining reliable alternatives for medical/dental use.

In this research study, the chlorhexidine digluconate 0.12% (Periogard®) was used as inhibitory control because it is considered a gold standard for oral cavity antiseptics. It has an antibiofilm action and can cause immediate reduction in 90% of the microorganisms. Its effect can remain from 30 seconds to 1 hour after mouthwashing, with a residual effect of until 7 hours [19]. However, despite its proven antimicrobial efficacy, its prolonged use causes alterations in the color of dental elements, restorations, prostheses, loss of taste, unpleasant residual taste in the mouth and, in a smaller number of cases, soft tissue burns, xerostomia, mucosa ulcerations, reversible swellings on the lips and parotid glands, scaly lesions, urticaria, dyspnea and anaphylactic shock [11]. Thus, the search for substances capable of inhibiting biofilms with fewer side effects, or rather without them, is justified. Among the phytotherapeutic agents most studied for dental use based on their antimicrobial, anti-inflammatory, healing and analgesic characteristics, clove, pomegranate, mallow, cat's claw and chamomile [20] stands out, the latter being the target of the present study. The *matricaria recutita* (chamomile) was the one chosen for this study due to its great consumption by the population as tea. It delivers a pleasant flavor, which would facilitate its use as a mouthwash and would still have little or no side effects when used for long periods. The goal of this research study was to verify the antibiofilm action of a chamomile formulation, in this case, the hydroalcoholic extract, as it has components with antimicrobial properties, such as the cis-trans-spiroethers, and coumarins, such as the herniarine and umbelliferone [21]. A concern was also to investigate published studies considering microorganisms in the planktonic phase presenting satisfactory results [22,23],

low cost and with potential to be used as a mouthwash. In the present study, to determine the minimum concentration of chamomile extract to be used in *C. albicans* and *E. cloacae* biofilms, as well as the effectiveness of the chlorhexidine formulation 0.12% (Periogard®), the broth microdilution test for these microorganisms when in the planktonic phase was carried out, which were respectively inhibited at 83 and 100 mg/mL and also by 0.12% chlorhexidine. In a disk-diffusion study with 25% (250 mg / mL) aqueous chamomile extract, Rahman and Chandra [24] reported a 24.16mm inhibition halo for *Candida albicans* in planktonic phase when compared to 33.26mm halo for chlorhexidine 2%. For *E. cloacae*, the inhibitory and bactericidal concentration were 10 µg/mL of chamomile essential oil, using microdilution in broth [25]. Both studies applied a chamomile formulation or a methodology different from those applied in this study, which could justify the difference of the results found.

Both *C. albicans* and *E. cloacae* were the microorganisms chosen for the tests due to their importance in oral diseases [3,9,10] ability to form biofilm [26,27], resistance [8,27] and the few studies related to the inhibitory activity of the hydroalcoholic extracts of chamomile when in biofilm. This work prioritized biofilm tests, since in this condition such microorganisms become more resistant to aggressions, increasing their capacity to install possible pathologies in the host. The efficacy of the hydroalcoholic extract of chamomile compared to chlorhexidine 0.12% was evaluated by two methods: colony-forming unit counts and Violet Crystal (VC).

The counting of colony forming units is a classic and reliable method, however it is very laborious, requiring culture medium and incubation time, besides counting and calculation to obtain the final result.

The quantification of biomass or cell density of viable cells with violet crystal has been a technique used in works with in vitro biofilm formation, being considered an sensitive indirect technique to demonstrate the formation of the same [28], besides being simple, economical and quick to achieve results.

According to the results achieved, there was a correlation between the cfu/mL count and the viable DNA density by the crystal violet technique. Considering the *C. albicans* biofilms, there was no significant reduction in the number of cfu/mL for all concentrations of the chamomile extract and chlorhexidine 0.12% in relation to the control, the same as with the crystal violet technique. In the *E. cloacae* biofilm, there was a significant reduction

in the number of cfu/mL only for the extract at 300 mg/mL and chlorhexidine 0.12%, whereas for the crystal violet technique, there was reduction with all the concentrations of the extract. Non-correlation in all assays may be due to the method of fixation applied, unlike other experiments that found high correlation. In the present study, fixation was performed with 10% formaldehyde, following the methodology described by Oliveira et al. [5] Freitas et al. [28] used thermal fixation (60° C for 60 minutes), based on Stepanovi [29] who reports that thermal fixation is the best method, but, also, gives the option of fixation using methanol for twenty minutes, followed by discarding and drying overnight at room temperature with the plate inverted.

There was a decrease in the number of cfu/mL for the *C. albicans* biofilms, with an increase in the concentration of the chamomile extract. Although the maximum reduction obtained with the extract at 300 mg/mL was similar to that caused by chlorhexidine 0.12%, there was no statistical difference with the control (untreated biofilm). The similarity with the reduction caused by chlorhexidine 0.12% was also obtained by the extract of chamomile 300 mg/mL for the biofilm of *E. cloacae*. However, this reduction was significant in relation to the control, demonstrating a better inhibitory action of both chamomile extract 300 mg/mL and chlorhexidine 0.12% for the *E. cloacae* biofilm.

In the reviewed literature, no experiment was found to test the action of chamomile with *Candida*

or *Enterobacter* species, or even another species of enterobacterium in biofilm. However, Agarwal et al. [30] applying different methodology from this work, among the essential oils tested against *Candida* species, the chamomile was not among the most effective ones.

Therefore, results achieved in this research study embrace new knowledge, as they demonstrate the inhibitory action of the hydroalcoholic extract of chamomile on *C. albicans* and *E. cloacae*. And, additional literature data considering its effective action in other oral microorganisms in biofilm, brings the possibility of performing further tests, aiming at its use as an oral antiseptic, even as an alternative to the use of chlorhexidine digluconate 0.12%.

CONCLUSION

The *M. recutita* extract 300 mg/mL significantly reduced the *E. cloacae* biofilm, but, not the *C. albicans* biofilm, both with a similar result to chlorhexidine 0.12% (Periogard®).

Collaborators

MA PEQUENO, implementation of methodology and writing. MR SILVESTRE, implementation of methodology and writing. I AMÊNDOLA, implementation of methodology and statistics. CRG SILVA and MVP LEÃO, outline of the methodology. SSF SANTOS, outline of the methodology and Orientation

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