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Preparation and evaluation of red propolis and nystatin cyclodextrin inclusion complexes against oral microbiome opportunistic microorganisms

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Abstract

The human oral cavity is colonized by a diversity of microorganisms living harmoniously. When an imbalance occurs in this population, also called dysbiosis, opportunistic microorganisms lead to oral infections. Nystatin, a traditional antifungal, and red propolis, a resinous mixture collected by honeybees from plant source, can be used to control oral infections. Using β-cyclodextrin (βCD) inclusion complexes with nystatin and red propolis may improve the solubility, stability and antimicrobial activity of these substances, making it possible to include these complexes in pharmaceutical formulations. This study aimed to obtain these inclusion complexes by solubilization and characterize and evaluate their antimicrobial activity against microorganisms from the oral cavity. The inclusion complexes showed loss of crystallinity and the infrared spectra showed interactions between the substances. The content of total polyphenol ranged from 4.32-4.62 mg/g, while the content of flavonoids was between 3.05-3.83 mg/g. The red propolis (Prop):βCD(1:1 and 2:1) complexes were the most active against *S.aureus*, *S.epidermidis*, *S.mutans* and *C.albicans* with Minimum Inhibitory Concentration (MIC) ranging between 32-256 μg/mL. The inclusion complex of nystatin (Nys):βCD(1:1) effectively inhibited *C.albicans* growth (MIC=4μg/mL). Interestingly, the association between Nys:βCD(1:1) and Prop:βCD(1:1 or 2:1) showed a synergic effect, improving antifungal activity when compared to red propolis complex alone.

Keywords: β-cyclodextrin; red propolis; nystatin; inclusion complexes; antimicrobial.

Practical Application: The complexes showed antimicrobial activity and can be included in different formulations.

1 Introduction

The oral microbiome is related to the various microorganisms that colonize the oral cavity (Deo & Deshmukh, 2019). Approximately 1000 species, among bacteria, fungi, virus and protozoa, inhabit the surface of teeth and the soft tissues of the oral mucosa (Aas et al., 2005; Yamashita & Takeshita, 2017). Current studies have reported that the oral microbiome composition changes during human development. The main bacterial genera found in a healthy oral cavity are *Streptococcus*, *Stomatococcus*, *Actinomyces*, *Lactobacillus*, *Propionibacterium* and *Haemophilus*. *Candida* species is the most representative fungi of the oral cavity, but other genera such as *Fusarium*, *Aspergillus*, *Cladosporium* and *Cryptococcus* can also be found (Willis & Gabaldón, 2020).

There is a homeostatic equilibrium between the host and microbiome population in a healthy individual. The microorganisms residing on the oral cavity prevent the establishment and uncontrolled growth of pathogenic or opportunistic species, and contribute to normal tissue and immune system development (Krishnan et al., 2017). However, some factors, such as diet, stress and some diseases, may affect the microbiome, leading to dysbiosis (Wade, 2016). This state is the result of an imbalanced proliferation of opportunistic microorganisms (e.g. *Staphylococcus aureus*, *Staphylococcus epidermidis*, *Streptococcus mutans* and *Candida albicans*), resulting in oral diseases such as periodontitis, dental caries, and candidiasis (Ramage et al., 2011; Rodrigues et al., 2016).

The clinical manifestations of oral candidiasis depend on their location, depth of epithelial invasion and host response (Hellstein & Marek, 2019). Patients may be asymptomatic or have symptoms related to burning, pain and dysphagia (Meira et al., 2017). Treatment of oral candidiasis can involve the use of topical formulations (nystatin, amphotericin, itraconazole, miconazole, clotrimazole) or systemic medicines (ketoconazole, fluconazole and itraconazole) (Kenechukwu et al., 2017; Ludwig et al., 2018; Scheibler et al., 2018). The choice depends on the patient's health status, the severity of the infection and the ability to use a topical agent. The use of topical drugs is the first choice when there are well-defined lesions. Systemic therapies are only justified in more severe cases. In topical therapy, the drugs used can be part of the

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group of polyenes or azoles and are available as solutions, oral suspensions, pastilles or tablets (Pappas et al., 2016).

Nystatin is a polyene that was discovered in the 1950s and has been used to treat oral candidiasis due to its fungistatic and fungicidal activity (Hazen & Brown, 1950; Robinson, 1955). This substance is a hygroscopic powder, with an odor of cereals and an unpleasant taste. It is sensitive to heat, light and oxygen. The effectiveness of nystatin depends on the time of direct contact with the oral mucosa affected by *C. albicans*. However, its low solubility in water and saliva can lead to ineffective sub-therapeutic concentrations (Groeschke et al., 2006). In general, nystatin used against oral candidiasis is marketed as an oral suspension. Due to the unpleasant taste and low contact time, the patient may not follow the treatment until the end, expelling the suspension before the correct time or using it without respecting the minimum dose regimen (Mims and Parker, 2006; Magalhães et al., 2021).

Propolis is a resin collected by bees of the species *Apis mellifera* and its natural function is to protect the hive from pathogenic microorganisms and other insects. The composition of propolis is heterogeneous, but it usually contains vegetable resins, essential oils, pollen, wood and earth debris and, in the hive, bees add salivary secretions and enzymes (Santos et al., 2020). Among the types of propolis cataloged in Brazil, red propolis has been the object of many studies. Red propolis is rich in phenolic compounds, triterpenes, isoflavonoids, benzophenones and naphthoquinones. One of the most usual source of red propolis is from the *Dalbergia ecastophyllum* plant, popularly known as "*rabo-de-bugio*" ("howler's tail"), found in the Brazilian northeast (Moise & Bobiş, 2020). Propolis is widely used in folk medicine and has several biological activities described as antioxidant (Guzmán-Gutiérrez et al., 2018), anti-inflammatory (Batista et al., 2018), antitumor (Borges et al., 2011), antiviral (Berretta et al., 2020), antibacterial (Yoshimasu et al., 2018), antiparasitic (Sena-Lopes et al., 2018), and antifungal (Siqueira et al., 2015).

The antifungal action of red propolis is attributed to the presence of phenolic acids, terpenes and flavonoids. Due to its pharmacological properties, associating nystatin and red propolis to improve the antifungal profile of topical formulations is a promising possibility. Despite the pharmacological advantages presented by nystatin and red propolis, the reduced solubility in water is a major drawback and requires the development of systems to optimize their solubility and increase stability, increasing the safety and effectiveness of these substances. In this context, the use of cyclodextrins (CDs) has been an important alternative used by the pharmaceutical industry and could also improve the taste and increase the therapeutic adherence of patients (Saokham et al., 2018).

CDs are cyclic oligosaccharides formed from the action of CD glycosyltransferases enzymes present in *Bacillus amylobacter* (Carneiro et al., 2019). CDs can be made up of six to eight glucose units and therefore are classified as α, β and γ-CD, respectively (Venturini et al., 2008). They are cone-shaped with a hydrophobic cavity and a hydrophilic external surface, being able to interact and accommodate lipophilic substances, thus forming so-called inclusion complexes with organic and inorganic molecules (Li et al., 2017). β-cyclodextrins (β-CD) are the most used, due to their low cost and the appropriate size of their cavity (Pinho et al., 2014).

In this study, we prepared inclusion complexes of nystatin and red propolis in β-CD and evaluated their antimicrobial profile and the effect of combinating these inclusion complexes against the *C. albicans* species, related to oral candidiasis in humans.

2 Materials and methods

2.1 Chemicals and reagents

The crude red propolis was donated by Bee Propolis Brazil and was collected in July 2017, in batch 42/17, in the state of Bahia, Brazil. Nystatin (Nys) was purchased from Antibiotice SA (Romania) and β-Cyclodextrin (βCD) was purchased from Wacker (Burghausen, Germany). Acid 3- (N-morfolin) propanesulfonic – MOPS, aluminum chloride, dimethyl sulfoxide (DMSO) 99.99%, Folin-Ciocalteu, gallic acid, quercetin and sodium carbonate were acquired from Sigma-Aldrich® (St Louis, USA). Acetone was obtained from Tedia® (Rio de Janeiro, Brazil), ethanol was obtained from Anidrol® (São Paulo, Brazil) and methanol was obtained from SK Chemicals® (Panguo-ro, Korea). Mueller Hinton Broth and Mueller Hinton Agar were acquired from HiMedia® (Mumbai, India), culture medium RPMI 1640 was obtained from Merck KGaA, (Darmstadt, Germany) and a flat-bottom 96-well titration plate was acquired from Kasvi® (Curitiba, Brazil). All other solvents and materials were analytical reagent grade or chromatographic grade.

2.2 Extract of red propolis

For the preparation of Brazilian red propolis extract, 27 g of propolis was extracted in a Soxhlet extractor for 12 hours at a reflux temperature (*ca.* 80 °C), using 500 mL of ethanol. Then, around 80% of total volume was evaporated to obtain an ethanolic extract, showing a final mass around 90 g. Concerning dry extract used to prepare the physical mixtures for comparision studies, the solvent was totally removed by rotary evaporation under reduced pressure.

2.3 Solubility curve of red propolis and nystatin

The solubility curve was performed in distilled water according to the method described by Higushi and Connors, with some modifications (Higuchi & Connors, 1965). 35 mg of red propolis extract and 30 mg of nystatin were added separately to 25 mL of aqueous solutions containing various concentrations of βCD (2-20 mM). The samples were shaken under magnetic stirring for 72 h in a dark place at room temperature. After that, the solutions achieved were filtered through a 0.45 μm hydrophobic polyvinylidene fluoride (PVDF) membrane to determine the total polyphenol content in the red propolis sample, using the Folin-Ciocalteu method, and the content of solubilized nystatin in the complexes was determined using UV spectroscopy. Absorbances were measured at 760 nm and 311 nm (nystatin), respectively. The value of K was calculated by Equation 1 where S_0 is the intrinsic solubility of the propolis/nystatin (the solubility in the aqueous media without CD) and

the Slope is the slope of the linear red propolis (Prop):βCD / nystatin (Nys):βCD phase solubility diagram.

$$K = Slope / S_0(1 - Slope) \quad (1)$$

2.4 Preparation of solid complexes of nystatin and red propolis

The inclusion complexes of Prop:βCD and Nys:βCD were prepared according to Pupe et al. (2013) with some modifications. The dry red propolis extract and nystatin powder were used to prepare a 1:1 mass proportion physical mixture with βCD for comparision studies. Inclusion complexes of Prop:βCD (1:1, 1:2 and 2:1 mass proportion) were obtained by adding 350 mg of red propolis extract solubilized in 25 mL of ethanol to the corresponding amount of βCD in 25 mL of distilled water, using magnetic stirring at 250 rpm for 72 hours. After the reaction time, the solvent was evaporated using a rotary evaporator and the samples were freeze-dried. Nys:βCD inclusion complexes (1:1, 1:2 and 2:1) were obtained by adding 500 mg of nystatin to the corresponding amount of βCD in 30 mL of ultrapure water. This solution was kept under stirring at 250 rpm for 24 hours and protected from light. After this period, the solution was frozen in liquid nitrogen and lyophilized for 72 hours. The sieving procedure was carried out using a sieve with 0.425 mm and measuring the final mass obtained to calculate the yield.

2.5 Characterization of cyclodextrin inclusion complexes

Incorporation yields

The incorporation yields were determined by the difference between the initial mass used and the final mass obtained after the lyophilization process. The results were represented by percentage.

X-ray powder diffraction (XRD), infrared spectroscopy (FTIR) and differential scanning calorimetry (DSC)

The X-ray powder diffraction analysis were carried out using a Shimadzu XRD-6100 X-ray diffractometer (Tokyo, Japan) using Cu-Kα radiation (k = 1.54 Å) that was operated at 40 kV and 30 mA. Measurements were obtained from 2º to 50º on the two theta scale.

FTIR spectra were collected using the Shimadzu IR-21 Prestige spectrophotometer (Tokyo, Japan) and each spectrum was acquired over the range from 500 to 4000 cm^{-1} using sample pads prepared with 1.0% (w/w) potassium bromide.

DSC analysis was performed using a Shimadzu DSC-60 calorimeter. Samples were heated from 30 °C to 250 °C at a rate of 10 °C/min under a nitrogen flow of 50 mL/min.

2.6 Analysis of secondary metabolites content in red propolis and flavonoid release from red propolis inclusion complex

Total polyphenol contents were determined by the Folin-Ciocalteu colorimetric method according to Swain and Hillis, with some modifications (Swain & Hillis, 1959). Thus, 100 µL of 25% w/v red propolis extract obtained before solvent evaporation were mixed with 2.5 mL of the Folin-Ciocalteu reagent (1:10) and 3 mL of 20% Na_2CO_3. Absorbance was measured at 760 nm after 2 hours incubation at room temperature in the dark. Total polyphenol contents were expressed by mg/g (gallic acid equivalents - GAE used for quantitative measurement). Total flavonoid contents in the extract and samples were determined using a method described by Santi et al. (2014) with minor alteration. For this, 100 µL of the same red propolis extract, 4.4 mL of ethanol and 0.4 mL of $AlCl_3$ 2% (m/v) in ethanol were added. After 30 min at room temperature, the absorbance was measured at 425 nm. Total flavonoid contents were calculated as quercetin (mg/g used for quantitative measurement) from a calibration curve.

It was performed a release assay with the most promising red propolis inclusion complex in cyclodextrin to observe if flavonoids could be released from the proposed inclusion complex. *In vitro* release experiments were performed using 200 mg of Prop:βCD (1:1) complex dispersed in 15 mL of distilled water and these suspension was added to a dialysis bag (cellulose acetate membrane; Sigma-Aldrich, St. Louis, USA, MO). The dialysis bags (n=4) were inserted in falcon tubes containing 30 mL of biorelevant medium (phosphate buffer pH 7.4) incubated at 37 °C with constant magnetic stirring (50 rpm) during 120 min (Abreu et al., 2016). At 0, 30, 60 and 120 minutes, 1 mL of medium were sampled from the release medium and the flavonoid content was determined using the methodology described above.

2.7 Biological assays

Microorganisms and growth conditions

The standard bacterial strains used in this study were *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228 and *Streptococcus mutans* ATCC 25175, obtained from the American Type Culture Collection (ATCC). The Muller-Hinton broth (MHB) and Muller-Hinton agar (MHA) (Becton Dickinson, Heidelberg, Germany) were used for susceptibility tests. The inoculum was prepared by selecting 5 colonies, suspended in sterile saline and quantified with a spectrophotometer (SpectraMax 190, Molecular Devices, Sunnyvale, CA, USA) at 570 nm to an optical density (OD) of 0.08 and 0.1, that related to 0.5 on the McFarland scale. Then, an aliquot of this inoculum was transferred to MHB to obtain a final concentration of 1.10^6 CFU/mL (Clinical and Laboratory Standards Institute, 2018). All strains were maintained in 10% glycerol stocks and stored at -80 °C. In this study, *Candida albicans* ATCC 24433 obtained from the American Type Culture Collection (ATCC) was also used. Fungal cells were cultured in Sabouraud dextrose agar (SDA) (Becton Dickinson, Heidelberg, Germany). The inoculum was prepared by selecting 5 colonies, suspended in sterile saline and quantified by spectrophotometer at 530 nm to an optical density (OD) of 0.08 and 0.1 that related to 0.5 on the McFarland scale. Then, an aliquot of this inoculum was transferred to RPMI 1640 medium with Mops (Merck KGaA, Darmstadt, Germany) to obtain a final concentration of 1.10^5 CFU/mL (Clinical and Laboratory Standards Institute, 2017). The culture was stored in Sabouraud dextrose broth (Becton Dickinson, Heidelberg, Germany) with 10% glycerol at -20 °C until use.

Minimum Inhibitory Concentration (MIC) using microdilution method

The cyclodextrin inclusion complexes of red propolis and nystatin were submitted to MIC analyses to determine the lowest concentration capable of inhibiting the visible bacteria and fungal growth. A series of three-fold dilutions of the complexes in concentrations from 1024 to 0.125 µg/mL were prepared in a 96-well microplate with a final volume of 100 µL in each well. Then, the dilutions were added to 100 µL of bacterial or fungal inoculum containing 10^6 and 10^5 CFU/mL, respectively, following CLSI recommendation (Clinical and Laboratory Standards Institute, 2017, 2018). A negative control was prepared using the inoculum without the complexes, whereas vancomycin and nystatin alone were used as a positive control. The microplates were incubated at 37 °C for 18-24 hours for bacteria and at 35°C for 48 hours for fungal growth. The results were determined by visual reading of the lower concentration that led to no turbidity. Experiments were performed in triplicate and the highest DMSO concentration used was 1%, which did not affect bacterial or fungal growth.

Minimum Bacterial and Fungal Concentration (MBC and MFC)

For MBC determination, after reading on MIC assay, an aliquot of 10 µL from wells where no visible growth was observed was cultivated on Petri dishes containing MHA. The plates were incubated at 37 °C for 24 hours and the number of colonies formed was counted. The MBC was defined as the lowest derivative concentration that was able to eliminate 99.9% of bacterial growth. The derivative was classified as bactericidal if no bacterial growth was detected in all triplicates (Novais et al., 2020). The MFC was performed according to Gómez-Sequeda et al. (2017), with some modifications. A total of 5 µL from the MIC wells where no visible growth was observed was transfered to microtubes containing 1 mL of RPMI 1640 medium with Mops. The microtubes were incubated at 35 °C for 48 hours. After this period, the results were evaluated according to the growth of fungal inoculum. If there was no growth in any microtube, the substance was characterized as fungicide, and if there was growth in all microtubes, it was classified as fungistatic.

2.8 Statistical analysis

All experiments were independently performed with at least three technical replicates. The results shown are the mean ± standard deviation (SD).

3 Results and discussion

3.1 Phase solubility studies

Phase solubility diagram is one of the most used methods to confirm the occurrence of inclusion complexes. From stability constant and its stoichiometry, it is possible to verify the solubility and stability increase of the drug in inclusion complexes by adding fixed amounts of drug in aqueous solutions of cyclodextrin in increasing concentrations (Brewster & Loftsson, 2007; Jacob & Nair, 2018).

The results indicated a linear relationship ($R^2 > 0.90$) between polyphenols—expressed as mg GAE—and the amount of βCD added, indicating that red propolis solubility increased linearly as a function of βCD concentration (Figure 1A). The stability constant was 438.18 M^{-1}, showing an adequate interaction between propolis and βCD. The solubility diagram obtained for the inclusion complexes between nystatin and βCD also indicated a linear relationship ($R^2 > 0.90$). Therefore, nystatin solubility increased according to the rise in the concentrations of βCD used (Figure 1B). The stability constant was 723.19 M^{-1}.

The curve acquired in this study was classified as A_L, where the increased linear solubility is proportional to the βCD concentration. Thus, the value of the slope was less than 1, indicating that at least a proportion of 1:1 between propolis or nystatin with βCD was obtained (Zeng et al., 2011). According to some authors, a stability constant between the range of 100-1000 M^{-1} is considered an ideal value (Manca et al., 2005; Santos et al., 2017). Thus, both samples of red propolis and nystatin inclusion complexes were within the range mentioned.

3.2 Characterization of inclusion complexes of red propolis and nystatin with β-cyclodextrin

Incorporations yields

Prop:βCD (1:2 and 2:1) and Nys:βCD (1:1) inclusion complexes presented incorporation yield values higher than

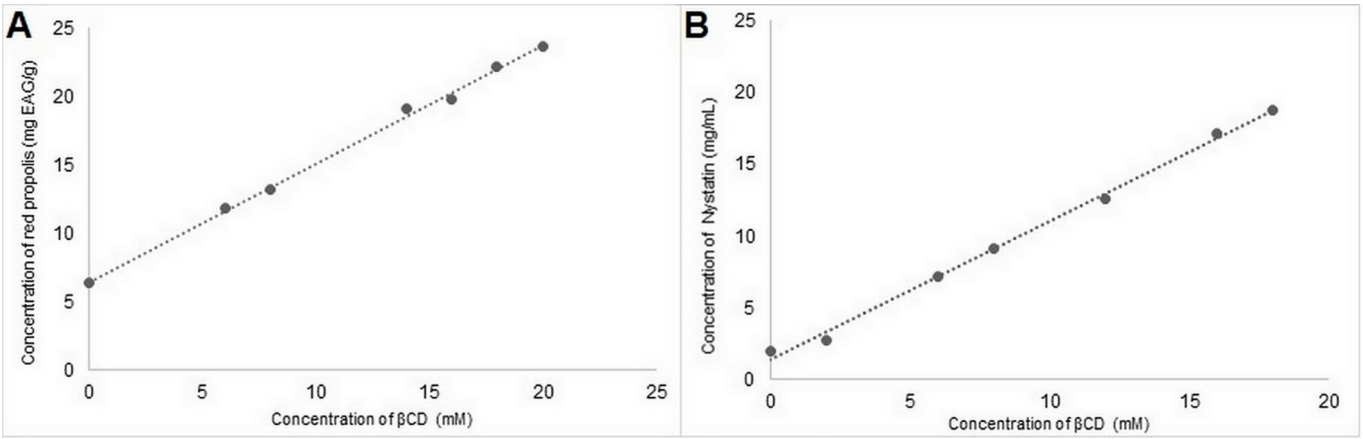

Figure 1. Phase solubility studies of: (A) red propolis with βCD and (B) nystatin with βCD. βCD = β-cyclodextrin

50%, with yields of 51.5%, 69.18% and 89.02%, respectively. The inclusion complex of Prop:βCD (1:1) showed a lower yield value of 40.79%. Nys:βCD (1:2 and 2:1) inclusion complexes showed yield values below 30%, and studies with these complexes were discontinued.

X-ray powder diffraction

The powder diffraction pattern of dried red propolis extract showed an amorphous structure, while nystatin and βCD showed sharp and intense peaks, indicating a crystalline structure (Figure 2) (Abarca et al., 2016). In a physical mixture, the intensity of crystalline peaks is maintained. Regarding the inclusion complexes, the peaks are smoothed due to the great decrease of crystallinity of the drug and cyclodextrin.

The reduction in peak intensity was due to the actual crystallinity loss or complete amorphization (Patel et al., 2007). This fact can represent an increase in water solubility and drug delivery (Riekes et al., 2010; Dan Córdoba et al., 2020). Significant differences are expected when the inclusion complex is formed due to the change in crystalline form.

Infrared spectroscopy (FTIR)

The infrared spectra of red propolis, nystatin and their complexes are shown in Figure 3. Some changes are observed: specifically, the interaction between the βCD band of type O-H and the hydroxyls from the propolis phenolic compounds in the 3600-3100 cm^{-1} region; the disappearance of the propolis band at 3070 cm^{-1} and 2852 cm^{-1}; the presence of lipids, flavonoids, amino acids, aromatic groups and aromatic rings, respectively in 1727 cm^{-1} and 1509 cm^{-1}, in the ratios 1:1 and 2:1; the disappearance of the propolis band in the region from 1727 cm^{-1} to 1200 cm^{-1}; and the reduction of the βCD band in 1157 cm^{-1} and 947 cm^{-1}, in the ratios Prop:βCD (1:2) and Nys:βCD (1:1). Complexes of nystatin and βCD also showed some modifications, for example, the disappearance of the nystatin band at 3013 cm^{-1} and 2975 cm^{-1} (C-H stretching vibrations), the reduction of the nystatin band at 1708 cm^{-1} (carboxylic acid/ether carboxile) and 848 cm^{-1} (CH=CH stretching vibrations), and a shift of the βCD band at 1420 to 1404 cm^{-1} (CH_2-CH_3) and 1068 to 1079 cm^{-1} (O-H stretching vibrations of nystatin). The spectra of the physical mixture of propolis with βCD and nystatin with βCD were almost the combination of pure components spectra and did not show any significant variations (Figure 3).

Infrared spectroscopy is useful to identify which vibrational modes of the drug and βCD are being changed in the inclusion complexes. Thus, changes in the characteristic bands of the complexes, such as shifting, broadening and disappearance can be indicative of complex formation (Cunha-Filho & Sá-Barreto, 2007; Lyra et al., 2010). In the 3600-3100 cm^{-1} region, the peaks are related to O-H bands of the glucose units of the βCD, showing no significant changes. Therefore, the band located at 3375 cm^{-1} observed on βCD, red propolis and inclusion complexes suggests the occurrence of intermolecular interaction between them, causing dispersion of propolis in the presence of βCD. This interaction is due to the combination of hydrogen bonds or van der Waals force (Calderini & Pessine, 2007; Li & Xu, 2010).

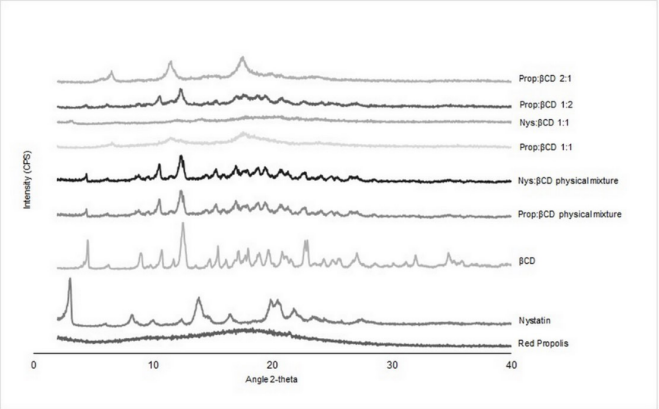

Figure 2. X-ray diffraction patterns of red propolis, nystatin, βCD pure, Prop:βCD physical mixture, Nys:βCD physical mixture, Prop:βCD (1:1, 1:2 and 2:1) and Nys:βCD (1:1). Prop = red propolis; Nys = nystatin; βCD = β-cyclodextrin.

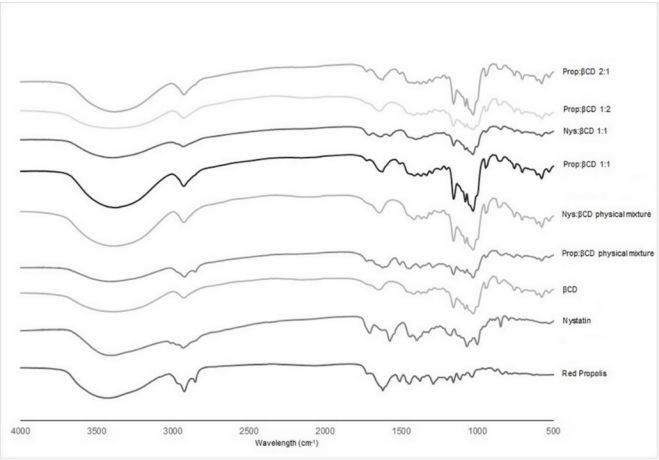

Figure 3. FTIR spectra of red propolis, nystatin, βCD pure, Prop:βCD physical mixture, Nys:βCD physical mixture, Prop:βCD (1:1, 1:2 and 2:1) and Nys:βCD (1:1). Prop = red propolis; Nys = nystatin; βCD = β-cyclodextrin.

The reduction of the band referring to the vibration of the C-O-C stretch by 947 cm^{-1} that involves α-1,4 type connections of β-CD, which are considered important due to its internal cavity, are indicative of complexation (Matioli, 2000). In addition, in the region of 1157 cm^{-1}, there was a small decrease in the bands in the complexes Prop:βCD (1:2) and Nys:βCD (1:1) due to the vibrational C-C connections of βCD (Rusa et al., 2001).

According to Zancanela and collaborators, during the process of incorporation of propolis in natural rubber latex membranes, no new band was observed when compared to pure propolis and membrane, evidencing a desirable behavior, since it would allow the release of propolis compounds from the membrane (Zancanela et al., 2019). This study showed similar results during the inclusion complex process with βCD.

Differential scanning calorimetry (DSC)

According to DSC results (Figure 4), the βCD and nystatin graphic showed endothermic peaks at 133 °C and 165 °C,

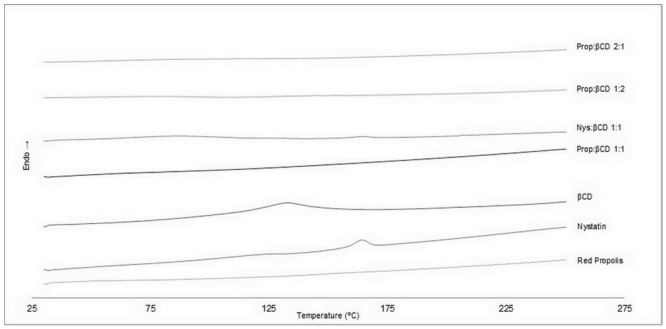

Figure 4. DSC curves of red propolis, nystatin, βCD pure, Prop:βCD (1:1, 1:2 and 2:1) and Nys:βCD (1:1). Prop = red propolis; Nys = nystatin; βCD = β-cyclodextrin.

respectively. Red propolis and all inclusion complexes did not show endothermic peaks, suggesting a possible interaction of βCD and propolis as well as nystatin, due to the disappearance of the melting point of βCD and nystatin.

The decrease or the disappearance of the endothermic peak can indicate loss of crystallinity of the samples. This effect can be related to the conversion of characteristic guest substance crystallin into amorphous, which is the result of the formation of the inclusion complexes (Mura et al., 1999; Bettinetti et al., 2002; Zingone & Rubessa, 2005; Bragagni et al., 2010; Ma et al., 2012). These results corroborate with the analysis found in the X-ray powder diffraction.

3.3 Red propolis total polyphenol and flavonoid contents and flavonoid release from red propolis inclusion complex

The content of total polyphenol determined in the samples of red propolis was 12.74 mg/g, while the inclusion complexes ranged between 4.62 to 4.32 mg/g (*Ca* 35%). The content of flavonoids of red propolis was 6.72 mg/g, and the complexes obtained ranged between 3.83 to 3.05 mg/g (*Ca* 51.91%). Therefore, the flavonoids were more complexed among the constituents of red propolis.

The quantification of polyphenols is very important, since various studies show that they are responsible for the pharmacological activity of red propolis. Flavonoids are another group present in red propolis that inhibits DNA, RNA and bacterial protein synthesis (Funari & Ferro, 2006; Alencar et al., 2007; Dzoyem et al., 2013; Freires et al., 2016). According to some authors, red propolis presented a variability of polyphenol and flavonoid contents around Brazil, ranging between 745.69 to 0.1763 mg/g and 140 to 0.00 mg/g, respectively (Alencar et al., 2007; Cabral et al., 2009; Righi et al., 2011; Batista et al., 2012; Hatano et al., 2012; Frozza et al., 2013; Mendonça et al., 2015a, b; Machado et al., 2016; Corrêa et al., 2017). The main effect that can affect the chemical composition of propolis is its geographic origin, because every region has its particularities—such as plant source, weather, soil, seasonality—and these factors can influence its composition and the contents detected (Teixeira et al., 2010; Sampaio et al., 2016).

The study of the release of red propolis inclusions was carried out focusing on monitoring the flavonoids present, given their lower solubility in water and, consequently, in the oral cavity. In the *in vitro* release assay 49.37 ± 5.90% (n=4) of flavoinoid was released from Prop:βCD (1:1) complex after 30 minutes with a cumulative amount of 49.83 ± 4.26% after 120 minutes, proving that flavonoid can be released from the proposed inclusion complex with a prolonged release profile. Therefore, the inclusion complex showed an initial burst effect followed by a prolonged release profile that is desired for a successful pharmaceutical treatment.

3.4 Results of microbiological studies

The antimicrobial properties of red propolis and nystatin associated with βCD were initially evaluated using the MIC method. According to the results, inclusion complexes of Prop:βCD, Nys:βCD and combinations of these inclusion complexes showed different antimicrobial profiles (Table 1).

The MIC of Prop:βCD for bacterial reference strains *S. aureus* ATCC 25923, *S. epidermidis* ATCC 12228 and *S. mutans* ATCC 25175 ranged between 128 to 512 μg/mL, within CLSI values (0.008–512 μg/mL) for commercially available antibiotics (Table 1). Notably, Prop:βCD in the 1:1 and 2:1 ratios (128 to 256 μg/mL) showed antibacterial effects up to twice better than the 1:2 ratio (512 μg/mL). The MIC value for non-complexed red propolis was 64 μg/mL (Table 1).

The antifungal activity was evaluated against *C. albicans* ATCC 24433. Again, Prop:βCD in the 1:1 and 2:1 ratios (32 μg/mL) showed better activity than the 1:2 ratio (128 μg/mL). The inclusion complex of nystatin and βCD in the 1:1 ratio (Nys:βCD 1:1) also showed promising antifungal activity (4 μg/mL) (Table 1). The inclusion complex Nys:βCD 1:1 was also tested against bacterial strains, but failed to show antibacterial activity, maintaining a result similar to that of non-complexed nystatin. The MIC value of the combination of Prop:βCD 1:1 (MIC = 32 μg/mL) and Nys:βCD 1:1 (MIC = 4 μg/mL) revealed that this association was positive to improve the antifungal activity against *C. albicans* ATCC 24433 (MIC = 2 μg/mL) (Table 1).

The analysis of the antibacterial and antifungal profiles of red propolis and nystatin complexed with βCD also included the determination of MBC and MFC. The MBC of Prop:βCD (1:1, 1:2 and 2:1 ratios) ranged from 256 to 512 μg/mL, except for the 1:2 ratio, which revealed a bacteriostatic effect against *S. epidermidis* ATCC 12228, similar to pure red propolis (Table 1).

The MFC of Prop:βCD 1:1 against *C. albicans* ATCC 24433 was 128 μg/mL, 4 times higher than MIC value (32 μg/mL). The Prop:βCD 1:2 and 2:1 ratios were classified as fungistatic. The nystatin complex (Nys:βCD 1:1) showed an MFC of 4 μg/mL, the same concentration of MIC (Table 1). The MFC values of combination between Prop:βCD in the 1:1 and 2:1 ratios with Nys:βCD 1:1 revealed that these associations have a fungicidal profile against *C. albicans* ATCC 24433, with the same concentration of MIC.

The microorganisms that are part of the oral microbiota are very diverse and colonize different niches within the oral

Table 1. Antimicrobial activity of inclusion complexes of red propolis and nystatin with β-cyclodextrin.

Inclusion complex	*S. aureus* ATCC 25923		*S. epidermidis* ATCC 12228		*S. mutans* ATCC 25175		*C. albicans* ATCC 24433	
	µg/mL		µg/mL		µg/mL		µg/mL	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MFC
Prop:βCD 1:1	128	512	128	512	128	256	32	128
Prop:βCD 1:2	512	512	512	-	512	512	128	-
Prop:βCD 2:1	128	512	128	512	256	256	32	-
Nys:βCD 1:1	ND	ND	ND	ND	ND	ND	4	4
Combination								
Nys:βCD 1:1 + Prop:βCD 1:1	ND	ND	ND	ND	ND	ND	2	2
Nys:βCD 1:1 + Prop:βCD 2:1	ND	ND	ND	ND	ND	ND	8	8
Controls								
βCD	-	-	-	-	-	-	-	-
Red propolis	64	-	64	-	64	64	8	32
Nystatin	ND	ND	ND	ND	ND	ND	1	1
Vancomycin	2	2	2	2	2	2	ND	ND

Prop = red propolis; Nys = nystatin; βCD = β-cyclodextrin; MIC = Minimum Inhibitory Concentration; MBC = Minimum Bacterial Concentration; MFC = Minimum Fungal Concentration; ND = not determined; - = without effect.

cavity, such as the palate, teeth and gums (Lamont et al., 2018). The bacterial species *Streptococcus mutans* is found in healthy oral microbiota, and fungal species like *Candida albicans* are also prevalent (Baker et al., 2017; Abranches et al., 2018). Although non-pathogenic, these microorganisms can cause oral disorders such as cavities and candidiasis. *S. aureus* and *S. epidermidis* can also use oral sites to invade the bloodstream and infect the heart, leading to bacterial endocarditis (Lockhart & Durack, 1999; Lima et al., 2019). In this study, we evaluated the effect of red propolis and nystatin complexed in βCD against these microorganisms.

Our results showed that both red propolis complexed in βCD (Prop:βCD) and the crude propolis extract showed activity against *S. aureus* ATCC 25923 and *S. epidermidis* ATCC 12228 (MIC = 64-512 µg/mL). Despite species of *Staphylococcus* spp. not usually being isolated in the oral cavity and, therefore, being considered part of a transient microbiota, immunocompromised patients or those with periodontal diseases present possible reservoirs for these opportunistic bacteria (Loberto et al., 2004).

Interestingly, Prop:βCD in different ratios (1:1, 1:2 and 2:1) showed bactericidal activity (MBC = 256-512 µg/mL). According to the literature, the antibacterial effect of red propolis occurs due to the presence of different phenolic compounds and flavonoids. *In vitro* studies have shown that these compounds cause structural damage to the bacterial membrane, or cell wall collapse, in the cytoplasm, leading to bacterial lysis (bactericidal effect) (Przybyłek & Karpiński, 2019). They also lead to growth inhibition and interruption of bacterial enzymatic activity (bacteriostatic effect) (Koo et al., 2002).

Red propolis is found in different regions of Brazil, and its antimicrobial activities can be influenced by factors such as temperature, rainfall cycles and site of extraction. Despite these factors, the crude red propolis extract used in this study showed anti-*Staphylococcus* and anti-*Streptococcus* activity (MIC = 64 µg/mL), as observed in previous studies that analyzed the activity of red propolis extracted from regions of the states of Sergipe, Alagoas and Paraíba against *S. aureus*, *S. epidermidis* and *S. mutans* (MIC = 50-200 µg/mL) (Hayacibara et al., 2005; Alencar et al., 2007; Lopez et al., 2015).

No studies in the updated literature evaluate the antibacterial effects of red propolis complexed with βCD. Interestingly, our results showed that the complexation was able to maintain the antibacterial profile of red propolis. Added to the increase in water solubility and the obtainment of a powder with good flow, enables its transformation into different formulations such as pastilles, tablets, toothpaste, films, among others. However, MIC values increased up to four times in the different analyzed ratios of Prop:βCD (1:1, 1:2 and 2:1), when compared to the red propolis extract. This difference may be related to the inclusion complex ability to reduce the lipophilic profile of propolis, changing its interaction with the bacterial cell wall and increasing propolis solubility. The main objective of complexing red propolis in CD is to obtain a solid compound that can be used as an active pharmaceutical ingredient (API). According to the *in vitro* release assay, a burst effect around 50% of red propolis was released from the cyclodextrin complex, followed by a slow and progressive release. As the fungicidal/bactericidal activity assay was performed at a specific time, it is expected that the red propolis has not been completely released from the inclusion complex, due to its prolonged release. However, with a prolonged use, it is expected to observe the effectiveness of the treatment as the red propolis will be released over the time.

The phenomenon of resistance to clinical-use antifungals decreases the therapeutic arsenal available for the treatment of these infections (Wiederhold, 2017). The expression of virulence factors such as the morphological transition from yeast to hyphae and the ability to form biofilms contribute to this scenario (Pukkila-Worley et al., 2009). The antifungal activity found for the crude extract of red propolis against *C. albicans* is important,

mainly because this species is the most prevalent in cases of oral candidiasis. There is a wide variability in the anti-*Candida* activity of red propolis extract. Siqueira et al. (2015), reported that the MIC of the red propolis extract against *Candida albicans* and non-*albicans* isolated from patients with chronic periodontitis ranged between 32 and 64 μg/mL. Our results showed MIC up to 8 times lower (MIC = 8 μg/mL) than results reported by Siqueira and collaborators.

The crude red propolis extract used in this study showed an MFC of 32 μg/mL, corroborating with previous studies that describe fungicidal and fungistatic activity for green and red propolis. This dual activity profile is interesting, because it helps the host's immune system to eliminate the fungus while preventing the pathogen from spreading to other areas of the body.

Despite the pharmacological advantages of red propolis, its poor water solubility may impact negatively its antimicrobial activity. Our study evaluated the complexation of red propolis with βCD, a cyclic oligosaccharide, capable of encapsulating hydrophobic substances and maintaining a hydrophilic external surface (Kost et al., 2020). The complexation of substances in cyclodextrins causes physicochemical changes in the complexed molecules that improve solubility, stability, and bioavailability, in addition to providing oxidative protection (Suvarna et al., 2017).

The reduction of water solubility can lead to ineffective sub-therapeutic concentrations of the drugs, including nystatin, an antifungal agent of the polyene group widely used to treat oral candidiasis. However, the hygroscopic feature and its sensitivity to light, heat and oxygen restrict the use of nystatin in topical applications (Borgos et al., 2006). In this study, using the solubilization complexation technique, nystatin was complexed with βCD in a 1:1 ratio and showed an MIC of 4 μg/mL against *C. albicans* ATCC 24433.

The comparison between the MIC of pure nystatin (MIC = 1 μg/mL) and that of the inclusion complex (MIC = 4 μg/mL) revealed an antifungal activity four times lower for the complex. According to the literature, cyclodextrin promotes a gradual release of the drug, which may explain the greater MIC of Nys:βCD 1:1. In the aqueous medium of the microplate on the MIC assay, this complex may have released the nystatin gradually, resulting in a higher MIC value. At the same time, the increase in solubility and stability of nystatin would justify the application of these inclusion complexes in therapy.

In Brazil, nystatin is marketed as a suspension for the treatment of oral candidiasis, which results in less therapeutic efficacy due to the low contact of the drug with the oral mucosa (Lyu et al., 2016). The complexation of nystatin with βCD can lead to the development of new pharmaceutical forms that would allow the drug to be more available in the oral cavity, in addition to improving palatability, since pure nystatin has an unpleasant bitter taste. These advantages caused by complexation may represent an increase in therapeutic effectiveness and greater patient adherence to treatment.

According to our results, the complexation of red propolis with βCD in the 1:1, 1:2 and 2:1 ratios showed an MIC between 32 and 128 μg/mL against *C. albicans* ATCC 24433. The best activity was obtained for Prop:βCD 1:1 and Prop:βCD 2:1 (MIC = 32 μg/mL). Molecules complexed in cyclodextrin can be fully or partially encapsulated (Duchêne & Bochot, 2016). Regarding the hypothesis that propolis complexation is partially occurring on Prop:βCD 1:2, the excess amount of βCD may be hindering the interaction of the red propolis phenolic and flavonoid compounds to exert antifungal activity against *C. albicans*.

Red propolis can also be used in association with commercial antifungals for a synergistic effect, leading to an increase in the treatment of infections. In this perspective, the combination of Nys:βCD 1:1 and Prop:βCD 1:1 showed an MIC of 2 μg/mL against *C. albicans*, a better result when compared to Nys:βCD 1:1 (MIC = 4 μg/mL) and Prop:βCD 1:1 (MIC = 32 μg / mL) evaluated separately, suggesting said synergistic effect.

The fungicidal activity of this combination was two times better (MFC = 2 μg/mL) than Nys:βCD 1:1 (MFC = 4 μg/mL) and sixty-four times better than Prop:βCD 1:1 (MFC = 128 μg/mL). Nys:βCD 1:1 in combination with Prop:βCD 2:1 also showed promising results when compared to Prop:βCD 2:1 evaluated separately, with a 4-fold reduction in MIC (32 to 8 μg/mL), and with fungicidal activity (MFC = 8 μg/mL) not detected previously for Prop:βCD 2:1 alone. The physical mixtures of nystatin βCD and propolis βCD were not tested, as βCD does not present antifungal or antibacterial activity.

4 Conclusion

In this work, the inclusion complexes Nys:βCD and Prop:βCD obtained by the solubilization method showed antimicrobial activity against opportunistic microorganisms from the oral microbiota (*S. aureus, S. epidermidis*, S. *mutans* and *C. albicans*). The combination of Nys:βCD 1:1 + Prop:βCD 1:1 and Nys:βCD 1:1 + Prop: βCD 2:1 showed promising synergistic effects against *C. albicans*. These results enable new studies that propose new pharmaceutical forms for red propolis and nystatin in order to promote greater stability, solubility and oral bioavailability, and could also improve the taste and increase the therapeutic adherence of patients for a more effective treatment against oral infections.

Conflict of interest

The authors declare that they have no competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Availability of data and material

The dataset analyzed during the current study is available from the corresponding author on reasonable request. Data generated during this study are included in this published article.

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