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Nanocapsules with glycerol monolaurate: Effects on *Candida albicans* biofilms

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ABSTRACT

Candida albicans does not only occur in the free living planktonic form but also grows in surface-attached biofilm communities. Moreover, these biofilms appear to be the most common lifestyle and are involved in the majority of human *Candida* infections. Nanoparticles can be used as an alternative to conventional antimicrobial agents and can also act as carriers for antibiotics and other drugs. In view of this, the aim of the study was develop, characterize and verify the anti-biofilm potential of GML Nanocapsules against *C. albicans*. The GML Nanocapsules showed mean diameter of 193.2 nm, polydispersion index of 0.044, zeta potential of −23.3 mV and pH 6.32. The microdilution assay showed MIC of 15.5 μg mL^{-1} to GML Nanocapsules and 31.25 μg mL^{-1} to GML. The anti-biofilm assay showed the significantly reduction of biomass of *C. albicans* biofilm treated with GML Nanocapsules while the GML does not exhibit effect. The kinetic assay demonstrated that at 48 h, the GML Nanocapsules reduce 94% of formed biofilm. The positive results suggest the promisor alternative for this public health problem that is biofilm infections.

1. Introduction

Biofilms are compact bacterial clusters that can adhere to many surfaces. They rapidly produce an extracellular polymeric matrix that is hard to penetrate, thus increasing the resistance of therapeutic drugs. Moreover, they are usually localized at sites difficult to reach, so current treatments are rarely successful. Currently, they are responsible for most microbial infections and the best option, besides prevention, is to remove the colonized tissue or implant [1–3].

The biofilm infections in hospital environment are a serious problem of public health and many methods has been used to try minimize or eliminate them. The great difficult lies in the fact of that many of these methods have important disadvantages, because lead to clinical complications and develop strains multi resistant [4].

Nanotechnology is one of the most prominent areas with the potential to tackle almost every aspect of microbial infections [2,5,6]. One of the main areas in focus is the development of therapeutic nanoparticles (NPs) for anti-biofilm applications. NPs can be synthesized through many different methods and approaches [7]. The reason why these molecules are so well studied and tested in the therapeutics of infections lies in their properties. Recently, this theme has been reviewed focusing on liposome and polymeric nanoparticles [1].

The glycerol monolaurate (GML) is a natural compound recognized as safe by *The Food and Drug Administration* (FDA). The antimicrobial potential of GML against many Gram Positive coccus in addition to *Bacillus anthracis* is known [8]. A previous study performed by Schlievert and Peterson, showed the ability of GML to inhibit the biofilm formation of three strains of *Staphylococcus aureus* including Methicillin resistant *Staphylococcus aureus* (MRSA) [9]. The present work is the first study that associates GML nanoparticles and biofilm, that despite promising, the use of GML is not expanded due the low solubility in water, leading to low bioavailability. The aim of present study was for the first time develop and characterize GML nanoparticles aiming the application on *Candida*

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albicans biofilms.

2. Materials and methods

2.1. Materials

The GML was purchased by *Seebio Biotech*, Inc®, Xangai, China. Sorbitan monooleate (Spam 80®), polysorbate 80 (Tween 80®) and acetone was purchased from Labsynth® (São Paulo, Brazil); capryc/caprylic triglyceride mixture was acquired from Brasquim (Porto Alegre, Brazil); the polymeric blende PMMA/PEG was supplied by Laboratory of Nanotechnology of Centro Universitário Franciscano (Santa Maria, Brazil).

2.2. Glycerol monolaurate nanocapsules

The GML Nanocapsules were produced according to the method described previously [10] with modifications. The aqueous phase was prepared with polysorbate 80 (0.194 g) and purified water (134 mL) at 40 °C under moderated stirring. In the organic phase, the GML (0.25 g) was solubilized with sorbitan monooleate (0.194 g), capryc/caprylic triglyceride (0.8 g), and polymeric blende PMMA-PEG (0.25 g) in acetone (67 mL) at 40 °C under moderated magnetic stirring. After solubilized, the organic phase was poured into de aqueous phase under magnetic stirring, being maintained for 10 min. The organic solvent and the water were evaporated in rotatory evaporator (Fisatom® Brazil) to adjust concentration to 1 mg/mL getting 25 mL of formulation. A blank formulation (Blank Nanocapsules) was developed in the same way as GML Nanocapsules (but without GML).

2.3. Characterization of GML nanocapsules

After preparation, the formulations were characterized as size and polydispersity index (PDI) by dynamic light scattering (DLS), zeta potential by electrophoresis in a Zetasizer Nano-ZS (Malvern Instruments, United Kingdom) and the pH was evaluated using potentiometer (Digimed®). Each parameter was evaluated in triplicated ($n = 3$) and results were expressed by average ± standard deviation (SD). The morphology of the nanocapsules were analyzed by transmission electron microscopy operating at 80 kV (TEM; Jeol, JEM 1200 Exll, Japan). Diluted suspensions (1:10 v/v in water) were deposited on specimen grid (Formvar-Carbon support films), negatively stained with uranyl acetate solution (2% w/v) and observed at different magnifications.

2.4. Microorganism

The strain *Candida albicans* (ATCC 14053) was obtained by American Type Culture Collection. This microorganism was maintained on culture medium with glycerol and cooled at −80 °C. The sample was unfrozen, inoculated on Brain Heart Infusion broth (BHI) and incubated for 24 h. After, it were seeded on Sabouraud agar and incubated for 24 h at 37 °C.

2.5. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC)

The MIC was performed by microdilution method on 96-well plate [11]. Different concentrations GML and GML Nanocapsules were add on wells containing Mueller Hinton broth (MHB). The positive control was considered the well with inoculum in MHB and negative control only MHB with saline. The assay was performed in triplicate. After the process, the plate was incubated to 24 h at 37 °C. After incubation, the assay was revealed with 2,3,5 triphenyl tetrazolium chloride. To determine the MBC, an aliquot of 1 μl was taken of each well, seeded on Sabouraud agar plate and incubated to 24 h. After, the colonies were identified and the lowest concentration which does not demonstrated microbial growth was considered the MBC.

2.6. Effect of GML and GML nanocapsules on microbial growth

Microbial growth curve was observed by inoculating the 96 well-plate with Mueller Hinton broth containing 1.5×10^8 CFU/mL of *C.albicans* and loaded with different concentrations of GML and GML nanocapsules (3.9–62.5 μg/mL). The plate was incubated at 37 °C for 30 h and the absorbance was reader at 600 nm [12].

2.7. Biofilm formation

The biofilm was formed according to the conditions previously optimized and described [13,14] with modifications. Fresh, exponentially grown culture of *C. albicans* was diluted to be 10^6 CFU/mL and 50 μL was added to flat-bottomed 24-well plates (Nunclon™ D surface, Nunc, Roskilde, Denmark), containing 500 μL of BHI broth and the plate was incubated in 37 °C, for 24 h.

2.8. Efficiency of GML and GML nanocapsules against biofilm developed

After formation of biofilm, it was performed the treatment and incubated for 24 h in condition of 37 °C according to Manner et al. [15]. The treatment was performed with 500 μL of a suspension containing 1 mg/mL of GML or GML Nanocapsules. A positive control was performed containing only BHI broth and the *C. albicans* strain while the negative control was just BHI broth.

2.9. Quantification of biofilm biomass

After the treatment, the supernatant was removed and washed four times with PBS and them, it was performed the quantifications. The result of biofilm treatment was measured fixing with 95%

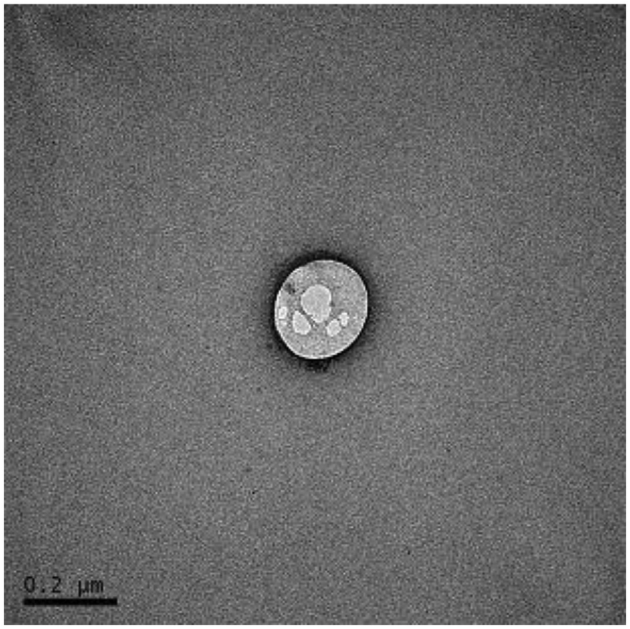

Fig. 1. GML nanocapsule in TEM.

of methanol and staining with 500 μL of 0.1% of crystal violet or 1% of safranin for 10 min at room temperature (RT). After incubation, the well-plates were washed with PBS and photos (Fig 4) were taken. Ethanol 95% was added to dissolve the coloring and after, transferred into other plate to measure spectrophotometrically at 570 nm to crystal violet and 492 to safranin in spectrophotometer (TP-Reader; ThermoPlate, Goiás, Brazil). The biofilm formation was determined by the difference between the mean OD readings obtained in the positive control (BHI broth and *C. albicans* strain) and the treatment with GML and GML nanocapsules.

2.10. Efficiency against biofilm formation

The GML and GML nanocapsules were tested to verify the ability in prevent the biofilm formation of *C. albicans*. The assay was performed in three replicates on 96 well-plates. It were used three sub inhibitory concentrations (0.5 × MIC, 0.25 × MIC and 0.125 × MIC) which were added together with microorganism. The experiment was performed in BHI broth for 24 h. After incubation, the biofilm was revealed such item above. Only BHI broth with *C. albicans* was considered control and the percentage of inhibition was calculated by $OD_{Test}/OD_{Control} \times 100$.

2.11. Kinetics of anti-biofilm activity on developed biofilm

Efficacy of GML and GML Nanocapsules were evaluated against *C. albicans* biofilm by the time-dependent killing assay. Biofilms of *C. albicans* were formed in microtube and treated with 1 × MBC of compound or formulation. Over a series of time intervals of 0, 3, 6, 12, 24 and 48 h, the anti-biofilm activity was measured with the safranin stain assay and the absorbance was reader in spectrophotometer (TP-Reader; ThermoPlate, Goiás, Brazil) at 492 nm. After coloration, the microtubes were washed 3 times with PBS, and was used ethanol to dissolve the safranin adhered in microtube. After discoloration, 300 μL were transferred to microplate for reading. The assay was performed in 3 replicates.

2.12. Biofilm treatment on glass slide and stained with Calcofluor White

A glass slide was inserted into petri dish, containing 10 mL of BHI broth. After, 50 μL of suspension containing *C. albicans* was added into the plate. The plate was incubated at 37 °C for 48 h. After biofilm formation, was performed the treatment with 1 × MBC of GML (62.5 μg/mL) and GML Nanocapsules (31.25 μg/mL). A dish without treatment was considered the Positive Control (only BHI + *C. albicans* suspension). After incubation, the glass slide was removing from dish, washed with PBS and dried at RT. Three drops of KOH (10%) and 3 drops of Calcofluor White Stain were dispersed in glass slide. After 1 min at RT, the glass slide was analyzed in Fluorescence Microscopy to observe the biofilm.

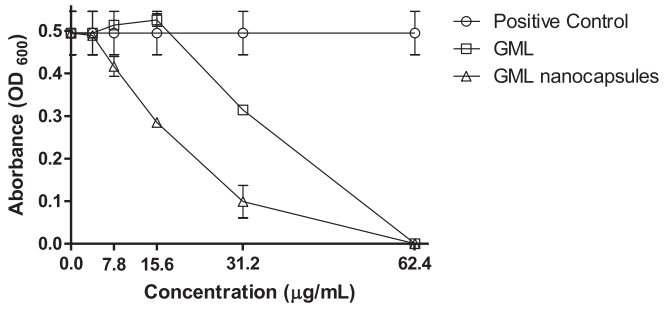

Fig. 2. Growth curve dependent of dose of GML and GML nanocapsules against *C. albicans*.

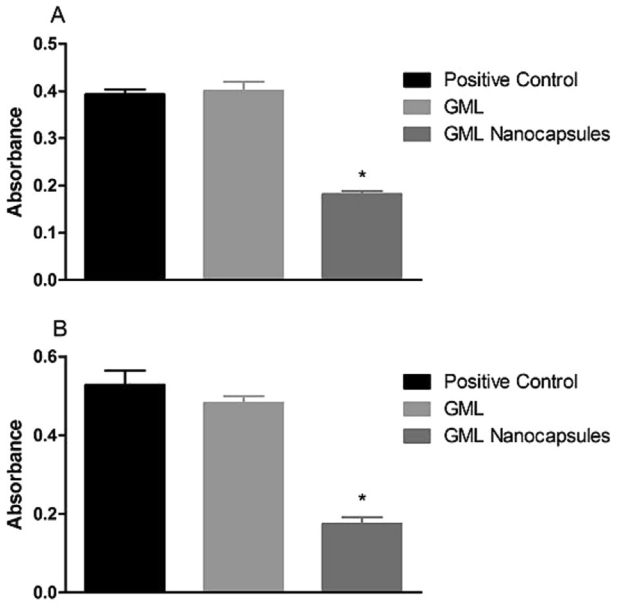

Fig. 3. Biofilm quantification by Crystal violet (A) and safranin (B) stain after exposure GML or GML Nanocapsules. Was used analysis of variance (ANOVA) followed by *Tukey* test considering values $p < 0.05$ statistically significant comparing with Positive Control. Data expressed on Average ± Standard deviation and absorbance reader with wavelength at 570 nm.

2.13. Statistical analysis

The results of microbiological assays were submitted to One-way analyses of variance (ANOVA) following by *Tukey* test with 95% of significance. The experiments were performed in 3 replicates (n = 3) except the biofilm assay which was carried in 2 replicates.

3. Results

3.1. Characterization of GML nanocapsules

After preparation, the formulation was evaluated as physical-chemical characteristic. The measurements showed values of pH, mean diameter, polydispersion index and zeta potential. The values were 6.32 ± 0.31 to pH, mean diameter about 193.2 ± 4 nm, polydispersion index of 0.044 ± 0.028 and zeta potential of −23.3 ± 3 mV.

The image of nanoparticle obtained by transmission electron microscopy was shown in Fig. 1.

3.2. MIC and MBC

The MIC and MBC can be visualized in Table 1. The Blank Nanocapsules was tested in the same way with GML Nanocapsules but without antimicrobial activity (data not shown).

3.3. Growth curve

The growth curve of *C. albicans* showed a difference of inhibition in relation to dose after 30 h of exposition. The result is according to MIC and MBC assays and is described in Fig. 3.

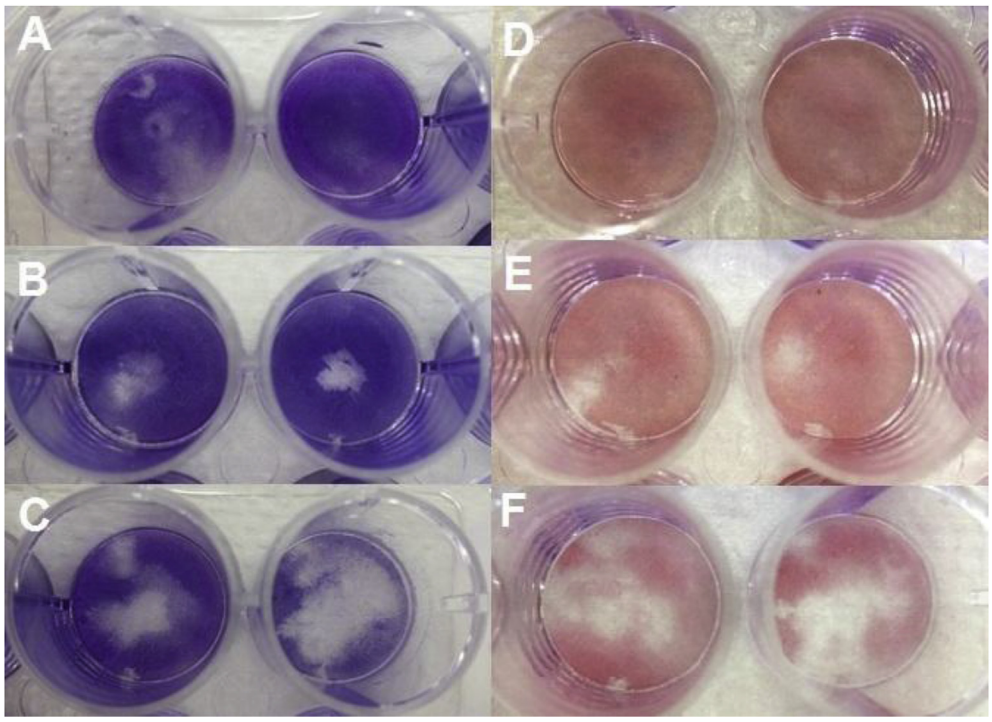

Fig. 4. Well-plate semi-quantification of biofilm by crystal violet (A to C) and safranin (D to F). Positive control (A,D), GML (B,E) and GML nanocapsules (C,F). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 1
MIC and MBC of GML and GML nanocapsules against *C. albicans*.

Microorganism	MIC (μg/mL)		MBC (μg/mL)	
	GML	GML nanocapsules	GML	GML nanocapsules
C. albicans ATCC 14053	31.25	15.5	62.5	31.25

3.4. Biofilm quantification by crystal violet and safranin

After stain procedure, the biofilm was quantified measuring the absorbance and comparing with positive control (*C. albicans* + broth). The result was described in Fig. 2. The Blank formulation was tested and do not demonstrate antibiofilm activity (data not shown).

3.5. Efficiency against formation of biofilm

After absorbance reading, it's possible observes that GML Nanocapsules inhibit the formation of biofilm at 0.5 × MIC concentration while the GML does not inhibit the biofilm formation. The results are described in Fig. 5. There was no inhibition of biofilms treated with Blank Nanocapsules (data not shown).

3.6. Kinetics of inhibition of formed biofilm

After 48 h, the GML Nanocapsules demonstrated capacity to eliminate virtually all biofilm (94%), while GML showed a lower effect. The result is described in Fig. 5.

3.7. Biofilm treatment on glass slide and stained with Calcofluor White

After staining, the glass slide was observed in Fluorescence microscopy and then took snapshots. The biofilm was observed in 400×. The images are demonstrated in Fig. 6.

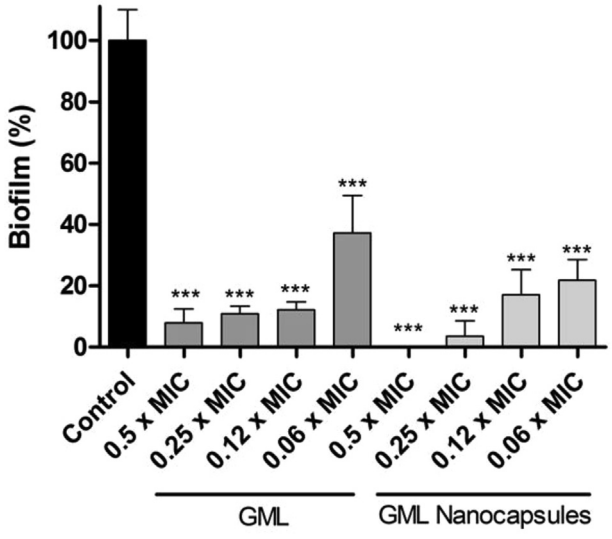

Fig. 5. Efficiency of biofilm inhibition of GML and GML Nanocapsules on many concentrations. Was used analysis of variance (ANOVA) followed by *Tukey* test considering values $p < 0.001$ (***) statistically significant comparing with Control.

4. Discussion

The synthesis of polymeric nanoparticles has been proposed to combat biofilm infections [16]. The polymeric nanoparticles would function as drug carriers that deliver the therapeutic molecule into the infected tissue, especially those that are water-insoluble, improving the effect on the biofilm [16]. The studies of nanoparticles against biofilm have demonstrated a promissory

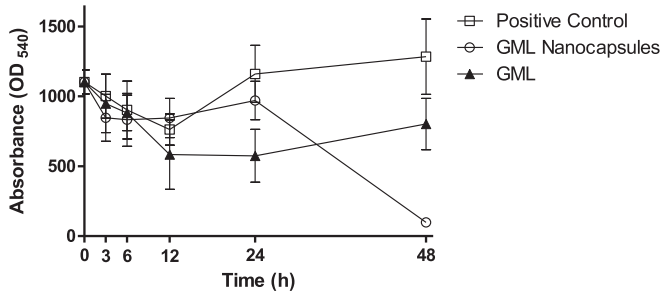

Fig. 6. Kinetics of inhibition of formed *C. albicans* biofilm with GML and GML Nanocapsules.

formation (Fig. 5).

In the kinetics assay, the GML Nanocapsules reduced approximately 94% of formed biofilm on 48 h while the GML reduced 46% in the same time. In the Calcofluor stain assay, the GML on concentration of 62.5 μg/mL it was not effective against *C. albicans* biofilm. The GML Nanocapsules on concentration of 32.25 μg/mL was able to significantly reduce the biofilm (Fig. 7).

Previous studies with development of nanoparticles with mean size of 220 nm and 260 nm were internalized by fungal cells due their reduced size have showed that due their reduced size, the nanocapsules could be internalized by fungal cells [29,30]. Moreover, the slow release of the GML could have had an important role

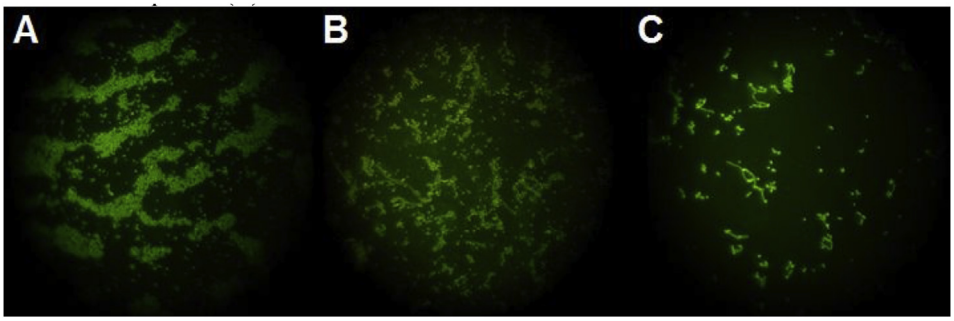

Fig. 7. Biofilm stained with Calcofluor White. Positive Control (A), GML (B) and GML Nanocapsules (C).

therapeutic alternative. Some kinds of drug nanoparticles are able to penetrate the barrier and eliminate biofilm. For example, only one dose of ciprofloxacin-PLGA nanoparticles reduced the *Pseudomonas aeruginosa* biofilm mass, size and live cell density by more than 80%, and repeated administrations prevented new formations [17]. A study with *Melaleuca alternifolia* oil nanoparticles showed anti-biofilm activity of *Pseudomonas aeruginosa*, also decreased the adhesion on epithelial cells and impaired the motility of microorganism, while the free oil do not showed effect [18]. Moreover, the nanoencapsulation of antibiotics resulted in better *in vitro* anti-biofilm activity compared to the free antibiotic [19,20].

In the present study, the formulations showed a milky bluish opalescent aspect (Tyndall effect) demonstrating success on the development [21]. After analysis in transmission electron microscope, images were produced and it was possible to verify the spherical shape and nanometric size proving the success of the development of the nanocapsule (Fig. 1).

Negative values to zeta potential are expected when used polymers containing grouping ester in structure, such as PMMA [22]. High values, in modulus of zeta potential, indicate that the nanoparticles have charges which allows the repulsion between other particles preventing the aggregation also predicting the formulation stability [23,24]. The obtained results of characterization in the present study corroborates with works related in literature which use polymeric blende in development of nanoparticles such carries and suggest homogeneity on size distribution [25–27].

A recent study of our group with GML Nanocapsules showed the antimicrobial activity against bee pathogen [28]. This is the first report which shows GML nanoparticles against biofilms. The assay with crystal violet demonstrated the high potential of GML Nanocapsules, against biofilm of *C. albicans* (Fig. 4C), while the GML don't showed significant effect being equal to the positive control (Fig. 4B and A). Studies performed by Schlievert et al. [9] demonstrated the capacity of GML on inhibit *Staphylococcus aureus* biofilms. The GML Nanocapsules demonstrated the ability to prevent biofilm in anti-biofilm activity throughout the time of the assay. In addition, a long time of release could help the dispersion of GML increasing the cellular penetration [31].

5. Conclusion

In conclusion, the study demonstrated the success of development of GML nanocapsules with acceptable values to predict a stable system. Moreover, the potential of nanocapsule against *C. albicans* was higher comparing the free GML. Furthermore, the anti-biofilm activity of GML Nanocapsules showed a therapeutic alternative to combat biofilms, considering that usual drugs do not penetrate into biofilm matrix and thus not being effective. Therefore, more studies must be performed to clarify the real mechanism of action of GML Nanocapsules and the role of nanostructuration on cell wall.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgment

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