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Topical delivery systems containing clotrimazole for the management of candidiasis: effect of different excipients and enhanced antifungal activity of nanovesicles

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Abstract

WHO classified *Candida albicans* as one of the four critical priority fungi for public health worldwide in 2022. Conventional topical formulations commercially available for the treatment of cutaneous candidiasis are associated with low drug bioavailability at the infection site and the lack of a sustained therapeutic effect. The main objectives of this work were to develop new topical administration systems of clotrimazole (CLT) and study the influence of surfactants on the antifungal inhibitory efficacy. Therefore,

the minimum concentration of CLT required to inhibit 50 % of growth (MIC_{50}) was determined, obtaining a value of approximately 15 ng/mL. A non-ionic emulsion type 1, Beeler base cream, hydrogel and liposomes containing CLT were designed, prepared, characterized and their antifungal activity against *C. albicans* was tested. CLT loaded liposomes were small in size (102 nm), homogeneous (polydispersity index = 0.3) and uncharged (+0.07 mV), showing higher antifungal activity against *C. albicans* than that of the commercially available cream Canesten®. Furthermore, the antifungal activity of CLT was reduced in combination with surfactants such as Tween-80/Span-80 or Brij-S10. Sodium lauryl sulphate showed a fungicidal effect that disappeared when formulated as part of the Beeler base cream.

Keywords: clotrimazole; liposomes; *Candida albicans*; surfactants; antifungal activity; topical administration.

1. Introduction

Fungal infections are benign pathologies that affect millions of people globally. Almost one billion people are estimated to suffer from skin, nail or hair fungal infections and over 150 million people have serious fungal diseases (Bongomin, et al. 2017). After the recent global pandemic of the coronavirus disease 2019 (COVID-19), their importance is on the increase as it has been reported that COVID-19 patients have a higher likelihood of suffering from fungal co-infections as an associated complication, with the main fungal pathogens being *Aspergillus* and *Candida* (Song, et al. 2020). Candidiasis is one of the most common fungal infections in humans and it is mainly caused by *Candida albicans* (Gómez-Gaviria and Mora-Montes 2020). This fungus has recently been classified by WHO as one of the four critical priority fungi for public health worldwide (World Health Organization 2022). As an opportunistic pathogen, *Candida spp.* can cause two types of infections, superficial (which include cutaneous and mucosal infections), and invasive systemic infections. In an immunocompromised host such as a COVID-19 patient, superficial candidiasis can overcome immunity and spread to the bloodstream, turning into invasive systemic candidiasis (Ahmed, et al. 2022).

Azoles are the primary antifungal agents used to treat superficial and invasive systemic mycoses, specifically imidazoles, which represent 65 % of the total consumption of antifungal agents, with clotrimazole (CLT) being one of the most commonly used ones because of its wide spectrum of antifungal activity, particularly against candidiasis (Yang, et al. 2008). For the treatment of systemic candidiasis, CLT is administered orally. However, because of its adverse effects following systemic administration, it is mainly used in the treatment of superficial candidiasis. Superficial candidiasis is the most common form (Arya and Rafiq 2022) and its treatment is based on topical administration forms, mainly due to the delivery of the drug to the specific site of action, the reduction of systemic toxicity and the elimination of pre-systemic metabolism. The problem with conventional topical marketed formulations, based on gels, powders, creams, solutions or foams of CLT, lies in the low drug bioavailability at the infection site and the lack of a sustained therapeutic effect, that makes the administration of very high and repeated doses necessary to achieve a local effect on the skin, which can lead to local or even systemic toxicity, such as hepatotoxicity,

cardiovascular disorders and drug interactions (Garg, et al. 2020). Furthermore, these formulations tend to accumulate in the stratum corneum, reducing their efficacy in the topical treatment of more invasive fungal infections (Erdal, et al. 2016). Another problem is that the efficacy of the formulations currently available on the market has been reduced due to the increasing percentage of strains that have developed drug resistance. For all these reasons, there is a need to develop new topical administration systems of CLT since it is well known that topical effectiveness seems to be strongly affected by the formulation (Clayton and Connor 1973; Sawyer, et al. 1975). In this sense, drug nanocarrier systems are gaining more and more relevance in the field of pharmaceutical technology since it has been observed that they improve the diffusion of active ingredients through the skin barrier.

Liposomes are currently one of the most successful nanocarrier systems for improving the topical efficacy of some drugs, proteins and natural active ingredients, as a consequence of their ability to penetrate the skin, which increases the biodistribution of the encapsulated substances (Castangia, et al. 2022; Perra, et al. 2022). In the case of CLT, its antifungal activity against *C. albicans* has been improved when it was formulated as a nanoemulsion (Soriano-Ruiz, et al. 2019), ethosomes (Akhtar and Pathak 2012) and three-dimensionally-structured hybrid vesicles (Manca, et al. 2019).

In the present work, new topical administration systems of CLT were developed. In this context, conventional topical formulations, such as emulsions (non-ionic emulsion type 1 and Beeler base cream), and novel topical delivery systems, such as hydrogels and liposomes, were designed, prepared and characterized. The antifungal activity of the formulations was tested and compared to the commercially available cream Canesten®, used as reference. Additionally, the influence of surfactants on the antifungal activity of CLT was studied since surfactants are essential raw materials in topical formulations that act by stabilizing them and promoting the penetration of drugs in the skin and the biofilm matrix.

2. Materials and Methods

2.1. Reagents

The following compounds were purchased from Acofarma (Madrid, Spain): clotrimazole, polyethylene glycol 400 (PEG 400), non-ionic self-emulsifying O/W base (Neo PCL® O/W), cetyl alcohol, white beeswax and glycerin. Sweet almond oil, butylhydroxytoluene (BHT), sodium lauryl sulfate (SLS), and carbopol 940 were obtained from Guinama (Valencia, Spain). Glucose, sabouraud dextrose agar (SDA), potassium chloride (KCl), sodium chloride (NaCl), acetonitrile (AcN) and methanol were obtained from VWR chemicals (Barcelona, Spain). The reagents purchased from Sigma-Aldrich (Madrid, Spain) were potassium phosphate monobasic (KH_2PO_4), di-sodium hydrogen phosphate (Na_2HPO_4), sodium phosphate monobasic (NaH_2PO_4) dimethyl sulfoxide (DMSO), RPMI 1940, Brij-S10, Brij-35, propylene glycol, tryptone water, Tween-80 and Span-80. 4-Morpholinepropanesulfonic acid (MOPS) and glutamine were obtained from Alfa Aesar (Kandel, Germany) and sodium hydroxide (NaOH) from Scharlau (Barcelona, Spain). Tween-60 and span-60 came from TCI (Tokyo Chemical Industry) and Canesten® was obtained from Bayer (Barcelona, Spain). Phospholipon 90 G was provided by Lipoid GmbH (Ludwigshafen, Germany).

2.2. Analytical method

CLT was quantified by means of a high performance liquid chromatography assay (HPLC) with ultraviolet (UV) detection at 260 nm. The HPLC equipment consisted of a quaternary pump SpectraSYSTEM P4000, an autosampler SpectraSYSTEM AS3000 and a spectrophotometric detector SpectraSYSTEM UV 6000LP. Data were processed through "Chromquest Chromatography Workstation Software Version 1.63". The column was a Waters "Nova-Pack" C18 (4 µm, 3.9 mm × 150 mm), and the mobile phase consisted of a mixture of acetonitrile, methanol and 25 mM sodium phosphate monobasic buffer, pH 4.6 (35/35/30, v/v). The injection volume was 25 µL, and the flow rate was 1 mL/min.

Content of CLT was analyzed in each formulation. To do so, CLT was extracted from samples with acetonitrile and, after centrifuging this mixture at 2000 × g for 5 min, the supernatant was injected into the HPLC system.

2.3. Method validation

The calibration curves (peak area versus nominal concentration) were constructed using a least square linear regression analysis for the calculation of the slope, intercept, and correlation coefficient. The accuracy (bias) and precision (relative standard deviation; RSD) of the assay were determined from CLT standards prepared at four concentrations (1, 10, 50 and 75 µg/mL).

The limit of detection (LOD) was estimated as the concentration of CLT giving rise to a peak whose height is 10 times the signal-to-noise ratio. The lower limit of quantification (LLOQ) was determined as the concentration of the lower standard with accuracy within 80–120 % and RSD within 20 %.

2.4. Topical administration systems of CLT

2.4.1. Emulsions

Two types of emulsions were prepared following the protocol indicated in the Spanish National Formulary (Spanish Agency for Medicines and Medical Devices (AEMPS) Sept 27, 2022): non-ionic O/W emulsion type 1 and Beeler base cream.

The oil phase of the non-ionic O/W emulsion type 1 was prepared with an O/W non-ionic self-emulsifying base (22 % w/w), sweet almond oil (3 % w/w) and BHT (0.02 % w/w); and the aqueous phase consisted of propylene glycol (5 % w/w) and a sufficient quantity of purified water (≈70 % w/w).

The Beeler base cream was prepared with cetyl alcohol (15 % w/w) and white wax (1 % w/w) as preservatives for the oil phase, while the aqueous phase was composed of propylene glycol (10 % w/w), SLS (2 % w/w) and a sufficient quantity of water.

For the formulation of the two types of creams, both phases were heated separately to a temperature of 70-75 ºC, and once melted, the aqueous phase was added to the oil phase while stirring to form the corresponding emulsion. Both creams were emulsions with the O/W sign, the first being a non-ionic emulsion while the second is anionic due to the incorporated surfactant.

In both emulsions, CLT was added in solution in DMSO (10 mg/mL) after sterilization by heat in an autoclave, with the final concentration in the creams being 0.5 mg/g. Empty emulsions (without CLT) were also formulated.

Additionally, Beeler base creams with some modifications in the composition of the oil or aqueous phase were prepared. Related to the oil phase, a cream without white wax was formulated, which was replaced by water. In other formulations, the cetyl alcohol was removed or the oil phase was eliminated completely and replaced by water, but no emulsions were obtained. The modifications in the aqueous phase included a cream without propylene glycol and a 10 % propylene glycol solution.

2.4.2. Hydrogel

To prepare the hydrogel, a weighed amount of Carbopol 940 (0.18 % w/w) was gradually added to water. The gel was left to stand at 25 ºC overnight for complete swelling. Afterwards the pH of the mixture was adjusted to 6-6.5 with 1 M NaOH. In this case, since the drug is a weak base, it would not be necessary to add PEG. However, as CLT is insoluble in water and soluble in PEG 400, CLT must be incorporated with the minimum amount of PEG and glycerin (which will represent 10 % w/w of the final volume). This mixture was added under stirring to the sterile gel to obtain a final concentration of CLT of 0.5 mg/g. An empty hydrogel (without CLT) was also formulated.

2.4.3. Liposomes

CLT-loaded liposomes were prepared mixing 50 mg of phospholipon 90G and 0.25 mL of a solution of CLT in DMSO (10 mg/mL). The mixture was homogenized with vortex and ultrasound for 30 minutes and thereafter hydrated with 4.75 mL of water warmed at 50 °C. Finally, the dispersion was sonicated with 2 cycles (2 cycles, 5 s on and 2 s off, 60 % amplitude) with an ultrasonic disintegrator (CY-500, Optic Ivymen system, Barcelona, Spain) to homogenize the preparation. To avoid a high increase in the temperature of the mixture as a consequence of the sonication process, the vial containing the mixture was placed in a container with water at room temperature. The temperature of the mixture at the end of the sonication was around 52 °C. The final concentration of CLT in liposomes was 0.5 mg/mL.

Empty liposomes (without CLT) were also prepared and used as reference in the characterization of CLT-loaded liposomes and in the antimicrobial tests.

2.4.3.1. Liposome characterization

Transmission electron microscopy (TEM) confirmed vesicle formation and morphology. The samples were stained with 2 % phosphotungstic acid aqueous solution and examined under a JEM-1010 (Jeol Europe, Paris, France) transmission electron microscope equipped with a digital camera AMT RX80 and the AmtV602 software, version 602.579 at an accelerating voltage of 80 kV.

Mean diameter (MD) and polydispersity index (PI) were determined by Photon Correlation Spectroscopy using a Zetasizer nano (Malvern Instruments, Worcestershire, UK). The same equipment was also used to measure the zeta potential (ZP) by means of

the M3-PALS (Phase Analysis Light Scattering) technique, which measures particle electrophoretic mobility.

2.4.4. pH measurement

The pH is a critical factor for all topically applied products prepared as aqueous liquid forms as it has an impact on the solubility of the molecule, determining the stability of formulations, their biological tolerability, and the activity of the molecule. For these reasons, pH values of the systems were analyzed using a pH meter (Fisher Scientific Accumet AE150, Madrid, Spain). To do so, different aqueous dispersions (10 % and 25 % w/v) were prepared.

2.5. Surfactants

The effect of surfactants on the antifungal activity of CLT was studied. To do so, aqueous solutions of anionic or non-ionic surfactants and CLT 500 µg/mL were prepared. Surfactants were chosen considering those employed in the emulsions of CLT previously formulated. The concentration of surfactants employed in the case of the non-ionic O/W emulsion type 1 (Brij-S10) and the Beeler base cream (SLS) was 2 % w/v. The non-ionic surfactants included in the formula of Canesten® were sorbitan stearate (Span-60) and polysorbate 60 (Tween-60). An aqueous solution with 1 % w/v of each one was prepared. Moreover, solutions of other non-ionic surfactants usually employed in the formulation of topical administration systems were tested, e.g. Brij-35 (2 %, w/v) and combinations of surfactants such as 1 % sorbitan monooleate (Span-80) and 1 % polysorbate 80 (Tween-80).

Aqueous solutions of surfactants without CLT were also prepared and used in the antimicrobial tests.

All of them were sterilized by heat in an autoclave after their preparation.

2.6. Microbial strains

The antifungal activity of CLT, both in aqueous dispersion and formulated as a topical administration system, and surfactants were tested against *Candida albicans* (CECT 1394). Cultures were kept for 24 h at 36 ± 1 °C. After 24 h of incubation, the fungal suspensions were diluted with PBS in order to obtain an adequate density expressed as colony forming units per milliliter (CFU/mL).

2.7. Antifungal susceptibility of *C. albicans* to CLT

The antifungal activity of CLT against *C. albicans* was evaluated by performing the broth micro-dilution method, using 96-well microtitration plates.

The yeast suspension was made by an initial dilution with tryptone water followed by another in RPMI 1940 according to the protocol of the European Committee for Antimicrobial Susceptibility Testing (EUCAST) (Subcommittee on Antifungal Susceptibility Testing (AFST) of the ESCMID European Committee for Antimicrobial Susceptibility Testing (EUCAST) 2008) and the M27-A2 procedure of the Clinical and Laboratory Standards Institute (CLSI) (Clinical and Laboratory Standards Institute (CLSI) 2017). The inoculum should be approximately 1-5 x 10^5 CFU/mL in the first one and 1-2.5 x 10^3 CFU/mL in the second.

100 µl of *C. albicans* inoculum was seeded in 96-well microtitration plates and treated with 100 µl of CLT at different concentrations (1000, 500, 250, 125, 60 and 30 ng/mL). To obtain the desired concentrations, CLT was first dissolved in DMSO (10 mg/mL) and subsequently diluted with water. In addition, a positive control was prepared in each test, in the absence of CLT. The negative control consisted of RPMI 1940 without inoculum. The 96-well plates were incubated at 35 ºC for 24 h or 48 h, according to the protocol followed (EUCAST or M27-A2, respectively) and the turbidity in each well was measured with a spectrophotometer (Hitachi U-2900, Tokyo, Japan) at a wavelength of 530 nm and compared with that obtained in positive growth control wells (with inoculum and without the assayed CLT concentrations) to determine the minimum concentrations of CLT required to inhibit the growth of microorganisms by 50 % (MIC_{50}). This value can also be estimated visually thanks to the absence of turbidity in the cases of inhibition. Moreover, samples were seeded in a Petri dish with SDA. After 48 hours of incubation, the colony forming units were counted to evaluate the MIC_{50} value.

2.8. Growth curves of *C. albicans*

To carry out this type of test, vials with 500 µl of aqueous solutions of CLT were used: 100, 50, 12.5, 1, 0.5 µg/mL and 60 ng/mL. All solutions were prepared from a stock solution of CLT in DMSO (10 mg/ml). In addition, a negative control containing 500 µl of water was used in each assay. 500 µl of inoculum containing 1-5 x 10^4 CFU/mL were added to all vials.

These tests were also carried out with the different CLT-based formulations (non-ionic O/W emulsion type 1, Beeler base cream, hydrogel and liposomes), empty formulations and Canesten®. For the preparation of the 500 µl of the different formulations, a 1/5 dilution with sterile water of the initial formulations was made, which contained a concentration of 0.5 mg/g. In the case of the marketed Canesten® (10 mg/g), a 1/100 dilution in water was made. Thus, the concentration of CLT in the diluted formulations was 100 µg/mL and the final concentration in the assay vials was 50 µg/mL. The assays also included a positive control composed by an aqueous dispersion of CLT (50 µg/mL), in which maximal *C. albicans* inhibition was expected.

All vials were incubated at 36 ± 1 °C, taking samples from each vial at 0, 9, 24, 33 and 48 hours. To collect them, 50 µl of each were diluted in 5 mL of phosphate buffered saline (PBS) and seeded in Petri dishes with SDA. The plates were kept in an oven for 48 h and the colonies observed were counted.

In addition, growth curves were performed in presence of CLT and different surfactants usually employed in the formulation of topical administration systems. As in the case of CLT-based formulations, a 1/5 dilution of the initial solutions of CLT and surfactants was made. The concentration of CLT and surfactants in the diluted solutions was the same as that assayed in the formulations. In these tests, sampling times were reduced to 0, 24 and 48 hours. The same assay conditions were used in the experiments performed with formulations obtained due to of different modifications of the Beeler base cream.

Furthermore, some of the samples were observed in a scanning electron microscope (SEM, S-4800 Hitachi, Tokyo, Japan). The samples were fixed with a solution of paraformaldehyde (2.5 %) and glutaraldehyde (0.5 %) (Karnovsky's fixative) and post-fixation with osmium (2 %). Thereafter, they were washed with distilled water and

dehydrated in a series of washes with ethanol (30, 50, 70, 90 and 100 %, v/v). The samples were filtered with a 0.1 µm pore polycarbonate filter. After drying by a critical point method with the automated Leica EM Cpd300 equipment, the samples were mounted on aluminum slides with double-sided carbon tape and shadowed with a nanometric layer of gold-palladium before visualization under the microscope. Some images were also obtained with an optical microscope.

2.9. Statistical analysis

Data are presented as mean ± standard deviation (SD). The Student's t test was used for comparisons of two groups. One-way analysis of variance (ANOVA) was used for comparisons of more than two groups; when statistically significant differences were found, Tukey's test was employed to determine which groups were statistically different. P values of < 0.05 were considered statistically significant. All calculations were performed with IBM SPSS Statistics 26 (SPSS Inc., Chicago, IL).

3. Results

3.1. Assay validation

A linear relationship was found between the CLT peak area and their concentrations in standards in the range of 1–100 µg/mL (Peak area = 14,499 x Conc (µg/mL) + 28,826, r > 0.999. LOD was approximately 0.2 µg/mL, and LLOQ was established at 1 µg/mL. Bias and RSD values of the method were lower than 5 % and 12 %, respectively.

3.2. Content of CLT and pH determination of topical administration systems

The concentration of CLT in each formulation was quantified and the results are shown in Table 1. As can be observed, the inaccuracy was lower than 6 % in all the topical systems designed.

Table 2 shows the pH values of empty and CLT loaded formulations. CLT is a weak base with pKa = 6.12 (Bones, et al. 2006) and its incorporation into the different formulations cause a slightly increase in the pH value. All formulations have a pH value in the range of 4 to 7, which is the physiological range of the skin and it suggests that they can be safely applied topically without irritation or dermatitis (Lambers, et al. 2006).

3.3. Liposome characterization

Liposomes loaded with CLT were mainly multilamellar, as detected by TEM analyses (Fig. 1A and 1B). The liposomes were small in size, spherical in shape, and slightly aggregated. Empty liposomes were also prepared in order to assess the effect of CLT on liposome assembly (Fig. 1C and 1D).

The physicochemical properties of liposomes were evaluated measuring the mean diameter (MD), polydispersity index (PI), and zeta potential (ZP) (Table 3). The empty liposomes were slightly smaller (91 nm) than those loaded with CLT (102 nm), but this difference was not statistically significant. The incorporation of CLT in liposomes did not modify the homogeneity of the systems. The surface charge of liposomes was

electrically neutral. Statistically differences in the ZP values were observed when CLT was incorporated into liposomes ($p < 0.001$), suggesting an encapsulation of the drug.

3.4. Morphological analysis of *C. albicans* by SEM and optical microscopy

Figure 2 shows different images of the growth stages of *C. albicans* obtained by SEM (A-C) and optical microscope (D-F) after 0, 24 and 48 hours of incubation. In these images, free-floating planktonic cells, reproducing by budding, are initially observed. After 24 hours, some cells clump together and start forming pseudohyphae, whose formation was evident at 48 hours and which can be considered the starting point of biofilm formation.

3.5. Antifungal susceptibility of *C. albicans* to CLT

The MIC_{50} of CLT solutions were measured while using the micro-dilution method according to the protocol of EUCAST and the M27-A2 procedure of CLSI. In Table 4, this value has been estimated counting colony forming units and in Table 5 measuring the turbidity. As can be seen, MIC_{50} values were lower when they were determined by counting colony forming units and the M27-A2 procedure (< 15 ng/mL).

3.6. Growth curves of *C. albicans*

Figure 3 shows the growth curves of *C. albicans* in the absence (negative control) and in the presence of different concentrations of CLT, with the final concentrations of the drug in the incubation medium being 50, 25, 6.25, 0.5, 0.25 μg/mL and 30 ng/mL. As can be observed, the growth inhibition is higher as the concentration of CLT increases. The highest CLT concentration (50 μg/mL) provoked an inhibition of 2.25 Log with respect to the negative control.

The growth curves of *C. albicans* in the presence of different formulations of CLT are shown in Figure 4A. The final concentration of all formulations in the incubation medium was 50 μg/mL. In this assay, a positive control composed by an aqueous dispersion of CLT was also tested and it was checked that it provoked the maximum inhibition of the growth. By contrast, negative control marked the absence of inhibition. Moreover, empty formulations were assayed and the results are shown in Figure 4B. These results were taken into account to calculate the real growth inhibition produced by each formulation of CLT and compare them with the ones obtained in the case of Canesten® at 24, 33 and 48 hours of incubation (Fig. 5). As can be observed, the non-ionic O/W emulsion type 1 was the least effective formulation, with an antimicrobial activity lower than the marketed cream Canesten®. No significant differences were found when the effectivity of the hydrogel was compared with Canesten®. The Beeler base cream also showed a similar effectivity as Canesten®, except at 48 hours, when it was significantly higher than that of Canesten®. However, the inhibition observed in the CLT loaded liposomes was higher at all times tested, with these differences being statistically significant.

Figure 6 shows the influence of different surfactants on the inhibitory activity of CLT at 24 and 48 hours of incubation in presence of *C. albicans*. No statistically significant differences were obtained in the growth inhibition of *C. albicans* when CLT was combined with Brij-35 or Span-60/Tween-60. However, the inhibitory potency of CLT was significantly reduced when it was tested with Span-80/Tween-80 or Brij-S10.

Finally, SLS showed complete growth inhibition, giving rise to values of 0 CFU/mL from 24 h of incubation, being even more powerful than the CLT dispersion. Therefore, decreasing concentrations of this surfactant (0.1-0.001 % w/v) were tested (Fig. 7). Higher SLS concentrations (0.1-0.01 %) continued to show a complete growth inhibition. After 24 hours of incubation in presence of SLS at a concentration of 0.005 %, the inhibition was higher than that observed in the dispersion of CLT (positive control) but not complete until 48 hours of incubation. SLS concentrations of 0.005, 0.003 and 0.002 % gave rise to a significant inhibition compared to that observed in the absence of this surfactant. SLS was the surfactant used in the Beeler base cream at a concentration of 2 % and it was checked that the fungicidal effect of SLS disappeared when it was formulated as a part of this type of emulsion (Fig. 4). For this reason, modifications of this cream were made and their inhibition capacity against *C. albicans* tested in order to elucidate the mechanism of action of this surfactant (Fig. 8). Firstly, the composition of the aqueous phase was modified, eliminating the propylene glycol, and the absence of inhibition of *C. albicans* was observed, which even seemed to favour growth. In the same way, the composition of the oil phase was modified, eliminating the white wax and without observing growth inhibition. However, when cetyl alcohol was removed from the oil phase or the oil phase was completely eliminated, a total inhibition of *C. albicans* was observed, as occurs when a 0.2 % SLS solution was tested. Finally, it was found that propylene glycol did not inhibit growth when administered in the absence of SLS.

4. Discussion

The search of formulation design strategies to improve the antifungal efficacy of CLT after its topical application has been the objective of many investigations in recent years. In this sense, different topical delivery systems loaded with CLT, such as two types of emulsions, a hydrogel and liposomes, were developed in this study.

CLT is degraded in strongly acidic and basic media and its optimal stability pH range is between 1.2 and 7.5 (Borhade, et al. 2012). In this regard, the pH values of the CLT loaded formulations were in the range of 4 to 7, which is within the above range of optimal stability.

Small liposomes containing CLT were obtained, with a mean diameter of around 100 nm and a polydispersity index value of approximately 0.3, which is the limit of the polydispersity index attributed to a homogeneous population of nanoparticles (Danaei, et al. 2018). Regarding the Z potential value obtained, the empty liposomes presented a negative value of -4.32 mV, while the loaded liposomes showed a value of +0.07 mV. Zeta potentials between −10 and +10 mV are considered to represent uncharged liposomes (Wang, et al. 2020). Some studies have revealed that the liposomal charge may contribute to their antimicrobial activity against planktonic and biofilm cultures. In this sense, uncharged liposomes loaded with ciprofloxacin or gentamicin showed more favorable interactions with planktonic *Pseudomonas aeruginosa*, while negatively charged liposomes significantly improved the activity of the drugs against the biofilm community (Alhariri, et al. 2017; Bandara, H M H N, et al. 2016). *C. albicans* has two forms of development: planktonic and in biofilms (Del Pozo and Cantón 2016). In the present study, the type of growth reproduced in the *in vitro* assays using 96-well microtitration plates was more similar to the planktonic than to biofilm one at least during the first 48 hours of incubation. However, after 48 hours the presence of

pseudohyphae, which can be considered the starting point of the biofilm formation, was evident.

Different MIC_{50} values of CLT against *C. albicans* were determined by various methods (by counting and measuring the turbidity) and protocols (EUCAST and M27-A2). The lowest value (15 ng/mL) was obtained with the combination of the counting method and the M27-A2 protocol. In general, the M27-A2 protocol resulted in a lower MIC_{50} value than that obtained with the EUCAST protocol. This fact was also observed in a study with fluconazole against *C. albicans,* in which the MIC_{50} value was found to be 16 µg/mL with M27-A2, while with the EUCAST protocol it was 32 µg/mL (Duarte, et al. 2010). However, in other studies, lower MIC_{50} values were obtained with the EUCAST protocol, in the case of fluconazole, itraconazole, posaconazole and voriconazole (Espinel-Ingroff, et al. 2005).

The lowest concentration used in the growth curve assays (30 ng/mL) was 2 x MIC_{50} and the highest concentration (50 µg/mL) was limited by the low aqueous solubility of CLT. In fact, a CLT concentration of 50 µg/mL was the maximum that did not give rise to a visible precipitation in the 96-well microtitration plate when it was prepared with RPMI 1940 medium pH 7 containing 0.5 % of DMSO. No differences were obtained in the antifungal activity of CLT 25 and 50 µg/mL, suggesting that higher concentrations of CLT would not enhance the inhibition of *C. albicans*.

When the C. albicans growth inhibition obtained with the different CLT-based formulations was compared with that observed with Canesten®, CLT loaded liposomes turned out to be the only formulation that showed a significant higher antifungal activity than Canesten® at all the assayed times. This result agrees with previous a study in which three-dimensionally-structured hybrid vesicles improved the cutaneous delivery of CLT in the treatment of topical candidiasis (Manca, et al. 2019).

The study of the effect of different surfactants on the antifungal activity of CLT against C. albicans showed that Brij-S10 (included in the non-ionic O/W emulsion type 1 formula) significantly reduced the growth inhibition provoked by CLT. This result explains the lower antifungal activity of the non-ionic O/W emulsion type 1 in comparison to that obtained with Canesten®. Although a complete growth inhibition was observed when CLT was combined with SLS at the same concentration as in the Beeler base cream (Fig. 6), the antifungal activity of this formulation was higher than that obtained with Canesten® only after 48 hours of incubation (Fig.7). Therefore, it was hypothesized that SLS alone has an antifungal effect or increases the antifungal activity of CLT and that the other components in the Beeler base cream formula counteract the effect of SLS. The antifungal effect of SLS alone gave rise to a growth inhibition of *C. albicans* (Fig.7). When the composition of the Beeler base cream was modified, it was observed that the antifungal effect of SLS disappeared as long as an emulsion was obtained. However, when the cetyl alcohol was removed from the oil phase or it was eliminated completely, a dispersion was observed and SLS provoked the growth inhibition of *C. albicans*. It seems that in an emulsion, SLS is retained in the emulsion droplets and its fungicidal effect disappears.

5. Conclusions

The overall results suggest that there is an influence of surfactants on the antifungal inhibitory efficacy of topical delivery systems loaded with CLT. In this sense, the

antifungal activity of CLT is reduced when is combined in formulations with Tween-80/Span-80 or Brij-S10 and the fungicidal effect of SLS disappears when it is included in an emulsion as a Beeler base cream. Furthermore, CLT has been suitably loaded in liposomes, thus facilitating their dermal delivery and interaction with epidermal cells, showing higher antifungal activity against *C. albicans* than that of the marketed cream Canesten®. These nanovesicles may be considered promising carriers for the treatment of topical fungal infections.

CRediT authorship contribution statement

Iris Usach: conceptualization, data curation, investigation, methodology, supervision, validation, writing - original draft. Paula Martínez-Álvarez: data curation, investigation, methodology. José Esteban Peris: conceptualization, data curation, supervision, writing - review & editing.

Declaration of Competing Interest

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

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Legend to figures

Figure 1. Transmission electron microscopy (TEM) images of CLT loaded liposomes (A and B) and empty liposomes (C and D).

Figure 2. Scanning electron microscopy (SEM) (A-C) and optical microscope (D-F) images of the negative control after 0, 24 and 48 hours of incubation with *C. albicans*.

Figure 3. Growth curves of *C. albicans* in the absence (negative control) and presence of different concentrations of CLT. Mean + SD, n=6.

Figure 4. Growth curves of *C. albicans* obtained in the presence of different formulations with (A) or without CLT (B). The curves corresponding to the positive (CLT 50 µg/mL) and negative (without CLT) controls are also shown for comparative purposes. Except in the negative control, the concentration of CLT in the incubation medium was 50 µg/mL. Mean + SD, n=6.

Figure 5. Growth inhibition of *C. albicans* provoked by different CLT-based formulations after 24 (A), 33 (B) and 48 (C) hours of incubation. Inhibitions were compared with that obtained with Canesten®. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 6. Growth inhibition of *C. albicans* provoked by CLT after 24 (A) and 48 (B) hours of incubation in the presence of different surfactants. Inhibitions were compared with that obtained with a dispersion of CLT. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

Figure 7. Growth curves of *C. albicans* obtained in the presence of different concentrations of SLS. The curves corresponding to the positive (CLT 50 µg/mL) and negative (without CLT) controls are also shown for comparative purposes. Mean ± SD, n=4.

Figure 8. Growth curves of *C. albicans* obtained in the presence of different modifications of the Beeler base cream. The curves corresponding to the positive (CLT 50 µg/mL) and negative (without CLT) controls are also shown for comparative purposes. Mean – SD, n=4.

Table 1. CLT content in different formulations (n = 4).

Formulation	Theoretical concentration	Observed concentration	Bias (%)

Non-ionic O/W emulsion type 1	500 µg/g	519.66 µg/g	3.93

Formulation	**10 %**	**25 %**
CLT Non-ionic O/W emulsion type 1	6.88 ± 0.11	6.71 ± 0.05
Empty non-ionic O/W emulsion type	16.22 ± 0.42	5.95 ± 0.38
CLT Beeler base cream	7.11 ± 0.01	7.03 ± 0.04
Empty Beeler base cream	6.83 ± 0.19	6.70 ± 0.07
CLT loaded Hydrogel	5.31 ± 0.01	5.02 ± 0.01
Empty hydrogel	4.35 ± 0.09	4.79 ± 0.01
CLT loaded liposomes	7.21 ± 0.12	7.12 ± 0.16
Empty liposomes	6.90 ± 0.13	6.79 ± 0.09

Beeler base cream	500 µg/g	520.58 µg/g	4.12
Hydrogel	500 µg/g	485.96 µg/g	-2.81
Liposomes	500 µg/mL	475.04 µg/mL	-4.99

Table 2. pH values of 10 % and 25 % w/v aqueous dispersions of empty and CLT loaded formulations. Mean values ± standard deviations (n=4).

Table 3. Mean diameter (MD), polydispersity index (PI) and zeta potential (ZP) of empty and CLT loaded liposomes. Mean values ± standard deviations (n=4).

	MD (nm)	PI	ZP (mV)
Empty liposomes	90.6 ± 4.8	0.30 ± 0.01	-4.32 ± 0.89
CLT loaded liposomes	102.2 ± 12.4	0.32 ± 0.04	0.07 ± 0.03

Table 4. Growth inhibition of *C. albicans* determined by counting the colony forming units (n=4). The MIC_{50} value obtained is shown in the last row.

	Growth inhibition (%)	
Concentration (ng/mL)	**EUCAST**	**M27-A2**
500	97.8 ± 1.8	99.5 ± 0.5
250	94.7 ± 2.5	94.7 ± 6.2

125	92.3 ± 5.1	91.9 ± 1.3
62.5	89.4 ± 4.1	83.7 ± 7.9
30	68.6 ± 10.7	78.2 ± 6.1
15	50.1 ± 11.5	63.6 ± 18.9
MIC_{50}	15 ng/mL	< 15 ng/mL

Table 5. Growth inhibition of *C. albicans* determined by turbidity measurement (n=4). The last row shows the MIC_{50} value or range of values obtained.

	Growth inhibition (%)	
Concentration (ng/mL)	**EUCAST**	**M27-A2**
500	91.1 ± 5.6	94.4 ± 5.0
250	89.9± 6.7	93.9 ± 1.3
125	75.4 ± 3.3	78.4 ± 6.8
62.5	71.0 ± 3.5	67.7 ± 0.9
30	41.1 ± 1.7	58.3 ± 0.5
15	29.1 ± 7.8	50.7 ± 1.1
MIC_{50}	41.5 ng/mL	15 ng/mL

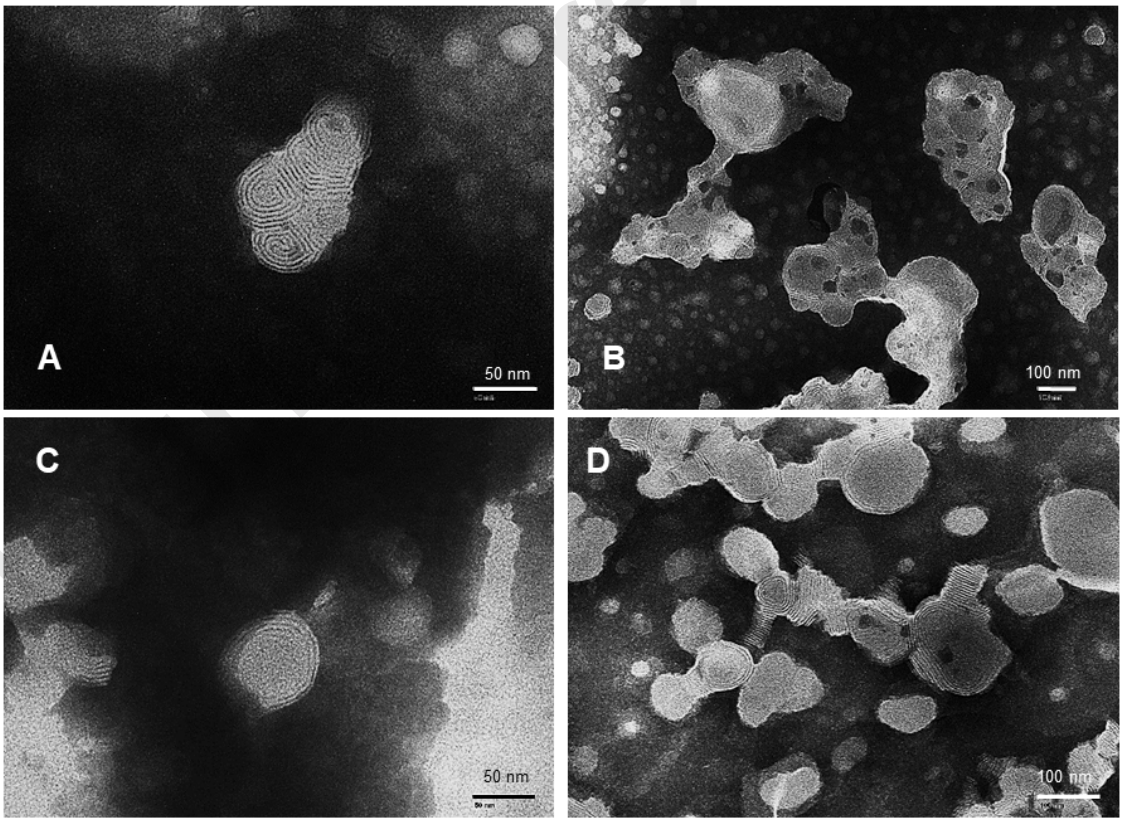
Author contributions

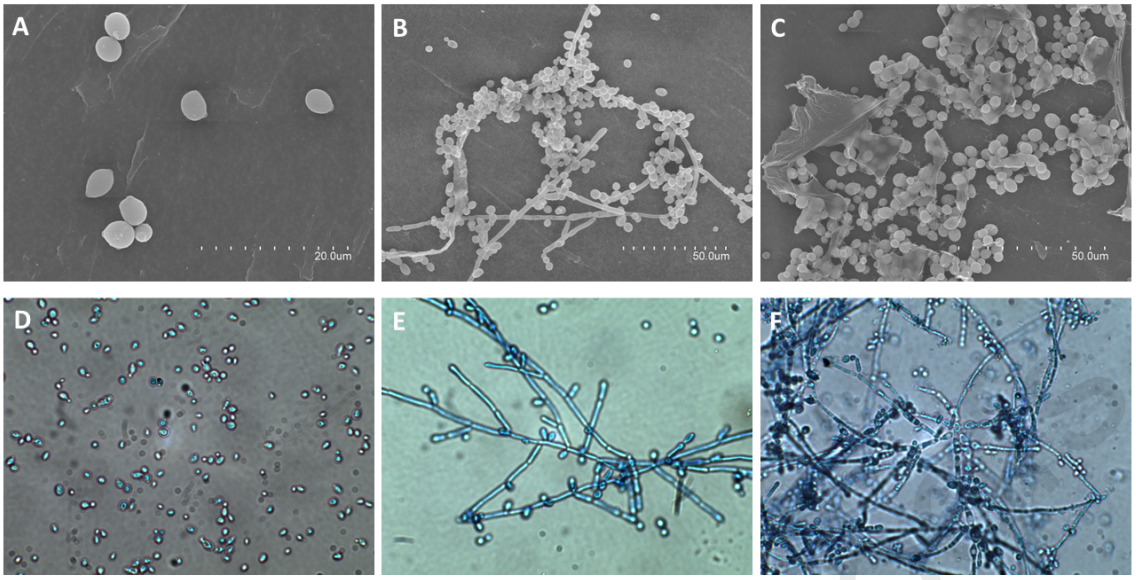
Iris Usach: conceptualization, data curation, investigation, methodology, supervision, validation, writing - original draft. Paula Martínez-Álvarez: data curation, investigation, methodology. José Esteban Peris: conceptualization, data curation, supervision, writing - review & editing.

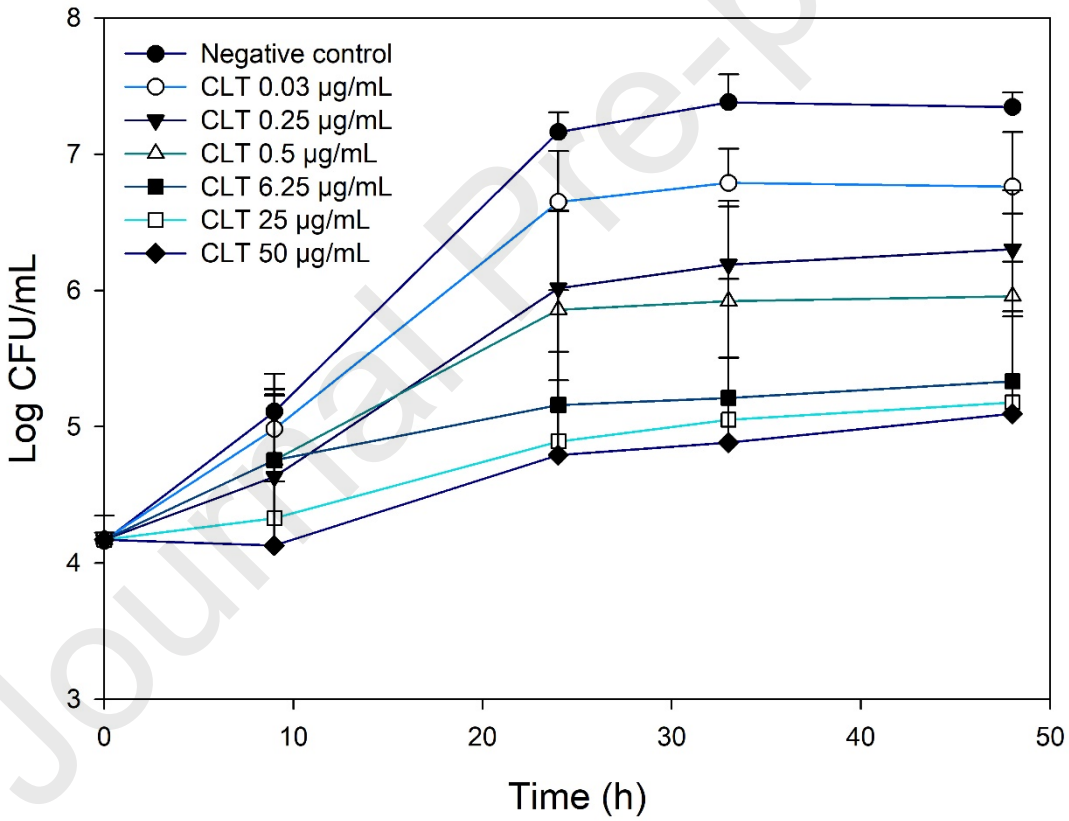
Declaration of interests

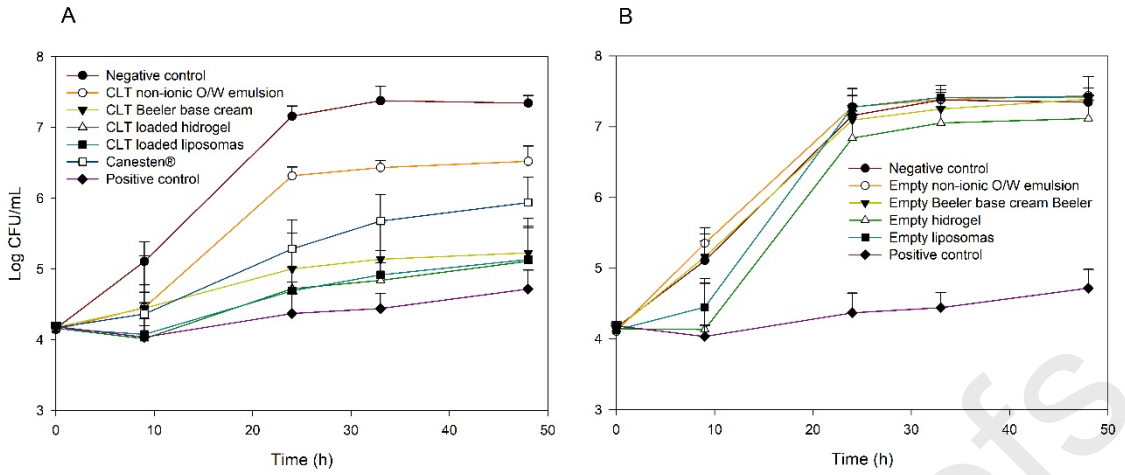
☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

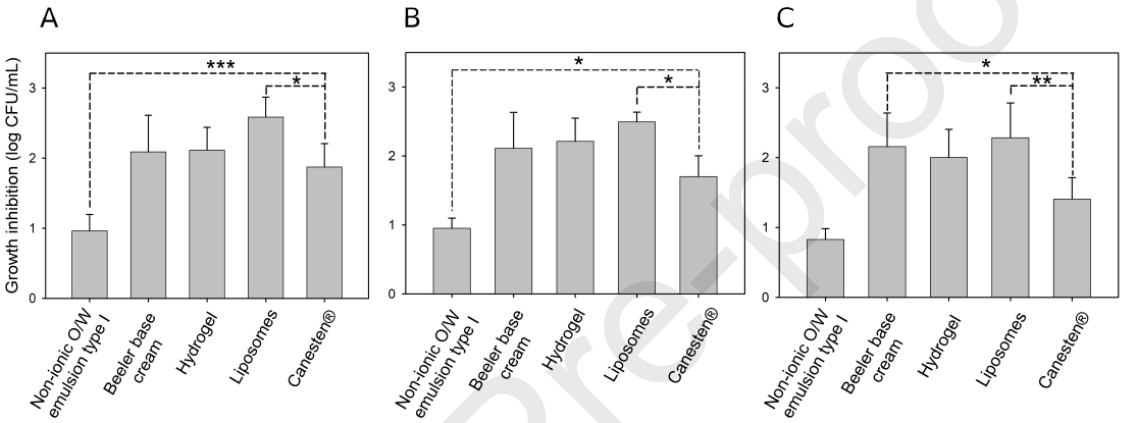
☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

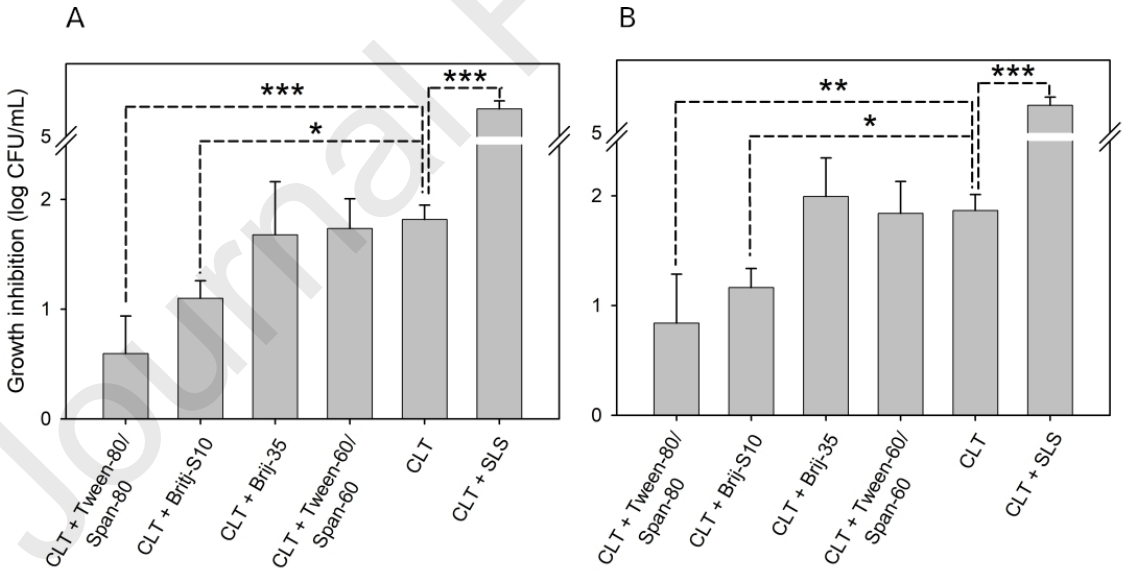


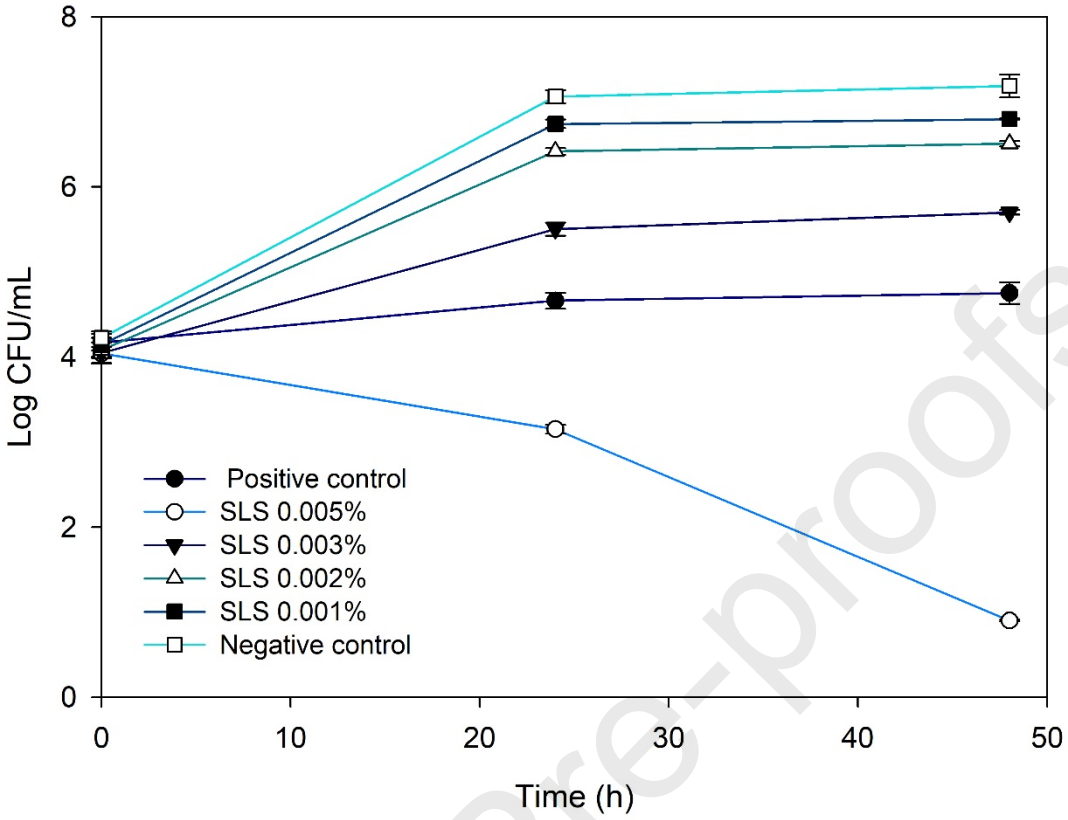


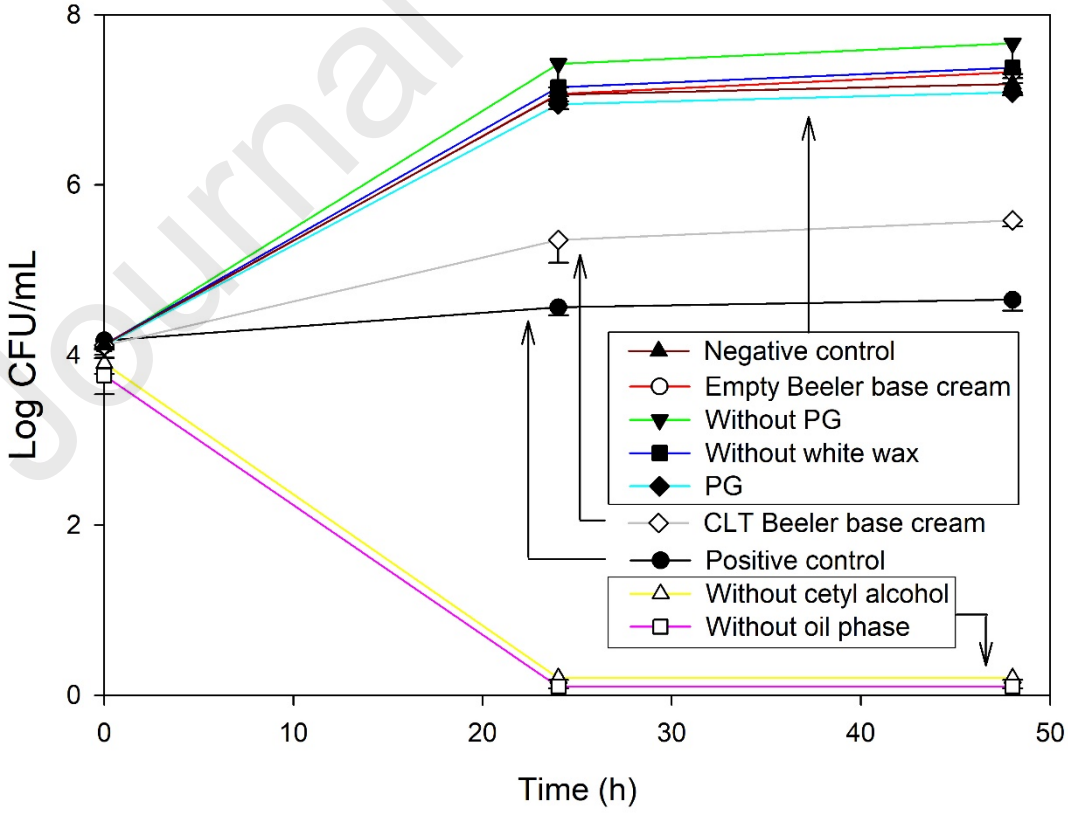


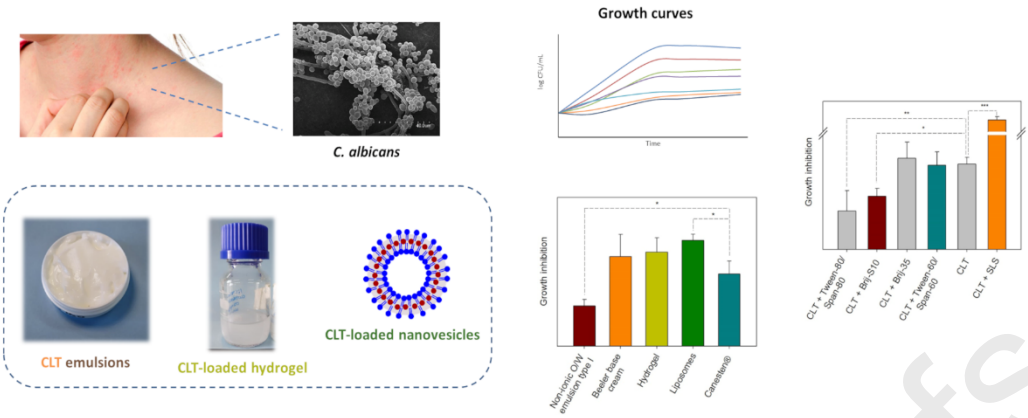












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