INTRODUCTION

Preservatives are used to increase the shelf life of products, preventing the growth of bacteria, filamentous fungi, and yeast, which can cause diseases or simply produce oxidative and/or hydrolytic enzymes that can promote physical, chemical, and pharmacological changes (Narayanan et al., 2017; Nemes et al., 2018), impairing the quality of the final product.

The effectiveness of the preservative may be compromised by interaction with active ingredients, excipients, and other factors such as pH value, partition coefficient, packaging material, and processing and storage temperatures (Santos, 2007; Elder, Crowley, 2017; Nemes et al., 2018). Excipients such as surfactants, macromolecules, and wetting agents can change the solubility and availability of the preservative, decreasing its efficacy (Akers, 2002; Allen Jr, Poppovich, Ansel, 2013).

Many incompatible formulations that result in preservative deactivation involve macromolecules such as cellulose derivatives, polyethylene glycols, and gums. In these complexes, especially those with paraben and phenol, the preservative activity is unavailable. The parabens are inactivated (partially or completely) by highly-ethoxylated compounds, cellulose derivatives, proteins, lecithin, and clays (Blanchard, 1980; Nemes et al., 2018).

The objective of this study was to determine the influence of nonionic surfactants on the effectiveness of preservatives used in emulsions containing high surfactant content. Mixtures of different concentrations were prepared between polyethoxylated (40) hydrogenated castor oil (PHCO) and polyoxyethylene sorbitan monooleate (PSO), with methylparaben, phenoxyethanol, methylyparaben, ethylparaben, propylparaben, and isobutylparaben (PMEPBI) blend, phenoxyethanol and benzoic acid (BP) blend, and phenoxyethanol and caprylyl glycol (PC) blend. Subsequently, the compatibility of the formulation ingredients and the effectiveness of the preservatives were evaluated by the challenge test. It was found that PHCO and PSO inactivated the antimicrobial action of methylparaben and PMEPBI. Paraben-free preservatives BP and PC had less influence on surfactants than systems containing parabens. When incorporated into microemulsions and nanoemulsions containing 40% and 20% surfactants, methylparaben and BP 0.2% and 0.5% were only effective against _Aspergillus niger_. The PMEPBI 0.2% was effective as a preservative in nanoemulsified formulations against _A. niger_, _Escherichia coli_, _Pseudomonas aeruginosa_, and _Staphylococcus aureus_. The results demonstrate that the efficacy of the preservative system in formulations containing nonionic surfactant excipients depends on the type of excipient, the components of the formulation, the preservative systems composition, the excipient to preservative ratio, and the availability in the formulation.

**Keywords:** Microemulsion. Nanocomposition. Formulation. Excipient. Stability
Emulsified systems such as nanoemulsions, microemulsions, and liquid crystals are dermal cosmetic preparations structured by the presence of polyethoxylated surfactants, which can decrease the effectiveness of the preservative in usual concentrations for conventional emulsion. Studies of the compatibility and effectiveness of the preservatives incorporated into nanoemulsified systems are not found in the literature.

Therefore, the objective of this study was to identify the polyethoxylated surfactant concentrations (polysorbate 80 and hydrogenated polyethoxylated castor oil) that affect the activity of preservative agents, both containing parabens and paraben-free, as well as to determine the effectiveness of these preservatives when incorporated into micro- and nanoemulsified systems for topical use.

**MATERIAL AND METHODS**

**Material**

Polyethoxylated (40) hydrogenated castor oil (Alkest® CSO400H) was kindly provided by Oxiteno (São Paulo, Brazil), polyoxyethylene sorbitan monooleate (Tween 80®) and methylparaben were purchased from Vetec (São Paulo, Brazil), caprylic/capric triglyceride (CCT) was purchased from AQIA (Guarulhos, Brazil), Phenonip® (blend of preservatives: phenoxyethanol, methylparaben, ethylparaben, propylparaben, butylparaben, and isobutylparaben) was purchased from Clariant (Muttenz, Switzerland) and used at 0.2% in formulations. Verstatil® BP (phenoxyethanol and benzoic acid blend) and Verstatil® PC (phenoxyethanol and caprylyl glycol blend) were purchased from Beraca (São Paulo, Brazil) and used at 0.5%.

For the microbial challenge test, each of the test formulations was inoculated with each of the following microorganisms: *Aspergillus niger* (ATCC 16404), *Candida albicans* (ATCC 10231), *Escherichia coli* (ATCC 8739), *Pseudomonas aeruginosa* (ATCC 9027), and *Staphylococcus aureus* (ATCC 6538).

**Inoculum preparation**

Microorganisms were grown in aerobic conditions. *E. coli, P. aeruginosa, and S. aureus* were grown for 24 h at 37 °C in Mueller-Hinton agar and later, the respective inocula were obtained according to CLSI (2015) to achieve a concentration of approximately $1.5 \times 10^5$ cells mL$^{-1}$. Yeast and fungal strains were cultivated on Sabouraud Dextrose agar at 30 °C for 48 h and 22 °C for one week, respectively, and each inoculum was obtained according to CLSI (2008a,b) to obtain a concentration of approximately $1.5 \times 10^4$ cells mL$^{-1}$.

**Compatibility analysis of preservatives and surfactants**

Using the modified Brazilian Pharmacopeia (Brazil, 2010) test procedure, compatibility analysis was performed using the mixtures: preservative + surfactant + culture medium; preservative + culture medium; surfactant + culture medium; and culture medium only. Fixed concentrations of the preservatives methylparaben (0.2%), PMEPBI (0.2%) and BP and PC (0.5%), and surfactants (PSO and PHCO) at 5, 10, 20, and 40 g L$^{-1}$ were used. Ten microliters of culture medium (broth) were put in sterile glass bottles, in duplicate, one for each organism, and inoculated with test microorganisms *A. niger, C. albicans, E. coli, S. aureus*, and *P. aeruginosa* ($10^5$ colonies or spores mL$^{-1}$ samples). The containers were incubated at room temperature (22.5 ± 2.5 °C).

The analysis was done initially and 7, 14 and 28 days after inoculation. Samples (50 µL) were inoculated by surface method in Mueller-Hinton agar (MHA) for bacteria (24 h at 37 °C) and Sabouraud Dextrose agar (SDA) for yeast (30 °C for 48 h) and fungi (22 °C for 1 week). The growth in terms of colony forming units per milliliter (CFU mL$^{-1}$) of each organism was determined.

**Effectiveness of preservatives incorporated in micro- and nanoemulsions**

The preservative effectiveness of methylparaben (0.2%), PMEPBI (0.2%), and BP (0.5%) in nanoemulsified systems with high surfactant content-type microemulsion (PHCO and PSO mixture 40%) and nanoemulsion (PHCO and PSO mixture 20%) were checked according to the Brazilian Pharmacopeia method (Brazil, 2010). The nanoemulsified systems were prepared according to Müller et al. (2015).
Inoculation of microorganisms

One milliliter of culture was put into plastic containers, one for each organism, and inoculated with the test microorganism to give a final concentration in the emulsions of $1 \times 10^5 \text{ CFU mL}^{-1}$. A 10 g sample was removed from each package for the zero-time count and the containers were incubated at room temperature ($22.5 \pm 2.5 ^\circ\text{C}$). Emulsions were analyzed at 7, 14, and 28 days.

Count of Colony Forming Units (CFU)

Ten grams of the samples were transferred to 90 mL of sterile soybean-casein broth (TSB) containing an inactivator (Tween® 80 30 g L$^{-1}$) and soya lecithin (3 g L$^{-1}$). The samples were homogenized, and 1 mL was inoculated by surface method in the culture medium (Soybean-Casein agar - TSA for bacteria and SDA for fungi and yeasts). The CFU mL$^{-1}$ was determined after an incubation period of 24-48 h at 32.5 ± 2.5 ºC for bacteria, 5-7 days at 22.5 ± 2.5 ºC for fungi, and 24-48 h at 30 ºC for yeasts.

Criteria for antimicrobial effectiveness

The antimicrobial effectiveness of the preservatives was determined according to Brazilian Pharmacopeia specifications for type 2 products (topical use). The calculated CFU mL$^{-1}$ was compared with the zero-time count and expressed in logarithmic reductions. By the 14th day, the number of CFU initially inoculated should be reduced by 2 logs and after 28 days there should be no increase in score compared to 14 days for bacteria. For fungi, on the 14th and 28th days there should be no increase in the number of CFU initially inoculated.

RESULTS AND DISCUSSION

The efficacy and compatibility of preservatives methylparaben, PMEPBI, BP, and PC in the presence of excipients PHCO and PSO were evaluated by reduction in microorganism growth. Using five microorganisms in the concentration of $10^5$ cells mL$^{-1}$ for bacteria, and $10^4$ cells mL$^{-1}$ for fungi and yeast, and analysis at 7, 14, and 28 days after incubation, preservatives were considered effective if after 14 days there was a decrease in microbial growth of 2 logs and if there was no increase in the number of CFU mL$^{-1}$ of fungi and yeast (Brazil, 2010).

Figure 1 shows the results of viable microorganism counts in the analyses with methylparaben (0.2%) and PMEPBI (0.2%) in the presence of nonionic surfactants PHCO and PSO, separately. In the analyses with methylparaben (10 g L$^{-1}$ and 5 g L$^{-1}$) and PSO, a decrease in the count for *C. albicans* was observed after 28 days of incubation. There was no reduction of 2 logs in microbial growth for other microorganisms.
The preservative composed of methyl, ethyl, butyl, propyl, and isobutyl parabens in phenoxyethanol (PMEPBI) showed incompatibility in the presence of nonionic surfactants because the microorganism growth was not reduced by 2 logs, as recommended (Brazil, 2010). However, the CFU mL⁻¹ number increased between 14 and 28 days.

Many preservatives used in pharmaceutical preparations cause interactions between preservatives and oil phase and/or emulsifying molecules or micelles. These interactions can be related to the lipophilicity of the preservatives and possible micellar solubilization. Above the critical micellar concentration, there is a decrease in preservative-free aqueous concentration and therefore a decrease in antimicrobial activity (Allen Jr, Poppovich, Ansel, 2013).

As described in the literature, parabens are inactivated (partially or completely) by sources of hydrogen ligands such as highly-ethoxylated compounds,

FIGURE 1 - Effect of preservatives methylparaben (M) (0.2%) and PMEPBI (P) (0.2%), in the presence of nonionic surfactants PHCO (A) and PSO (T) at 5, 10, 20, and 40 g L⁻¹, on microbial growth of five tests microorganisms after 28 days of incubation.
polysorbates (PSO 3%), and other compounds such as cellulose derivatives, proteins, and lecithin (Blanchard, 1980; Sheskey, Cook, Cable, 2017). The effect of different concentrations of PSO on methylparaben, phenoxyethanol, and chlorocresol against *P. aeruginosa* was investigated by Kurup *et al.* (1991), who found that in the product with concentrations greater than 0.5%, the antimicrobial effect was eliminated.

The preservative BP (0.5%) in the presence of nonionic surfactants (PHCO and PSO), separately, was effective. Logarithmic reduction (2 logs) in the count of viable microorganisms after 28 days of incubation was observed (Figure 2). PC (0.5%) in the presence of nonionic surfactants (PHCO) was effective against the fungus *A. niger*, as it could reduce the concentration of CFU mL⁻¹ at 14 and 28 days compared to the initial inoculum.

**FIGURE 2** - Effect of preservatives BP and PC (0.5%), in the presence of nonionic surfactants PHCO (A) and PSO (T) at 5, 10, 20, and 40 g L⁻¹, on microbial growth of five test microorganisms after 28 days of incubation.
In combination with the emulsifier PHCO in the concentrations analyzed (5, 10, 20, and 40 g L\(^{-1}\)), PC (0.5%) did not provide effective conservation because there was an increase in growth between 14 and 28 days of incubation for \(C.\) \textit{albicans} (concentrations of 40, 20, and 10 g L\(^{-1}\)), \(E.\) \textit{coli} (concentrations of 40, 20, and 10 g L\(^{-1}\)), \(P.\) \textit{aeruginosa} and \(S.\) \textit{aureus} (concentrations of 40, 20, 10, and 5 g L\(^{-1}\)) (Figure 2).

PC in the presence of PHCO (at 5 g L\(^{-1}\)) showed effectiveness for \(C.\) \textit{albicans} (Figure 2), which remained at the same concentration as the initial inoculum on day 14 and reduced 2 logarithms on the 28\(^{th}\) day. For \(E.\) \textit{coli}, CFU was decreased by 2 logs on the 14\(^{th}\) day compared to the initial inoculum but remained with the same score on the 28\(^{th}\) day at 5 and 10 g L\(^{-1}\) of surfactant.

The CFU concentration of the \(C.\) \textit{albicans} inoculum did not change between the 14\(^{th}\) and 28\(^{th}\) days in the presence of PC (0.5%) combined with PSO at 20 g L\(^{-1}\) (Figure 1). Phenoxyethanol, the main ingredient of BP and PC, has a wide spectrum of action against Gram-negative and Gram-positive bacteria and low efficiency for fungi. Association with another preservative to enhance the antimicrobial action is recommended. BP (phenoxyethanol in combination with sodium benzoate) was effective in the presence of nonionic surfactants.

The lowest power preservation of the PC in the presence of PHCO may be related to the incompatibility of phenoxyethanol, the main preservative, with nonionic surfactants (Sheskey, Cook, Cable, 2017). In this preservative, phenoxyethanol is associated with caprylyl glycol emollient, which by itself is not considered a preservative, but together with a preservative may potentiate the antimicrobial action. This potentiation is of greatest interest in the case of more resistant microorganisms like fungi and Gram-negative bacteria (Pinto, Kaneko, Pinto, 2015).

BP showed more preservative activity than the other preservatives studied (PC, methylparaben, and PMEPBI) in the presence of the nonionic surfactants for the studied microorganisms.

After the results of preservative and excipient compatibility tests, the preservatives chosen to incorporate in emulsion formulation were: methylparaben, a combination of methyl, ethyl, butyl, isobutyl, and propyl parabens in phenoxyethanol (PMEPBI), which are frequently used in compounding pharmacies and in the cosmetic industry, and phenoxyethanol (and) sodium benzoate (BP) and phenoxyethanol (and) caprylyl glycol (PC), a promising paraben-free preservative alternative.

Microemulsion and nanoemulsion formulations containing 0.2% methylparaben or 0.2% PMEPBI or 0.5% BP were challenged with five microorganisms separately and at predetermined periods (7, 14, and 28 days). Samples of these formulations were diluted and inoculated in a solid medium for counting viable microorganisms present in the formulations. The results of the challenge test of emulsions containing the preservatives are shown in Figure 3a-f.
Compatibility and efficiency of preservatives in emulsive cosmetics containing high surfactant content

**FIGURE 3** - Preservative efficacy analysis of different preservatives in micro- and nanoemulsions for five microorganisms.

3a,b: methylparaben (M) at 0.2%; 3c,d: phenoxyethanol (and) benzoic acid (BP) at 0.5%; 3e,f: phenoxyethanol (and) methylparaben (and) ethylparaben (and) propylparaben (and) butylparaben (and) isobutylparaben (P) at 0.2%. 
Inoculum standardization was carried out using the McFarland 0.5 nephelometric scale, which recommends a concentration of $10^5$ CFU mL$^{-1}$. Thus, after standardization for turbidity, the standard plate count was performed to check the density of the microbial load and determine whether the value was within the recommended values. At zero time the microorganism counts were on the order of $10^5$ CFU mL$^{-1}$, the desired value in a challenge test, because this way it verifies the initial amount of inoculum added. At zero time the contact between the microorganism and the preservative present in the formulation is extremely fast, not allowing a decrease in microbial concentration.

According to the criteria established for antimicrobial efficacy (Brazil, 2010), the formulations analyzed are classified as category 2, i.e., topical products formulated in an aqueous vehicle, nonsterile nasal products, and emulsions, including those applied on mucous membranes. For these products, there must be a 2 log reduction in CFU mL$^{-1}$ for bacteria on the 14th and 28th days and the counts must not increase relative to the 14th day. For yeasts and fungi, on the 14th and 28th days there should be no increase in the number of CFU mL$^{-1}$ initially inoculated.

Preservatives methylparaben (0.2%) and BP (0.5%) were not effective for nanoemulsion and microemulsion formulations (Figure 3a-d) because they do not provide logarithmic reduction (2 logs) in the viable microorganism count after 14 days of incubation. According to Sheskey, Cook and Cable (2017), methylparaben showed low antimicrobial activity when used alone, but its activity may be enhanced using parabens in a mixture with other synergistic effects. The combinations of methyl, ethyl, propyl, butyl, and isobutylparaben are often used, as PMEPBI. One can also add other excipients, such as propylene glycol (2-5%), phenylethyl alcohol, and EDTA.

The result obtained with BP incorporated in the emulsion oil phase was unexpected because, in the studies of effectiveness against nonionic surfactants, logarithmic reduction (2 logs) in the count of viable microorganisms after 28 days of incubation was observed.

Preservative effectiveness in the presence of excipients alone and incorporated into micro- and nanoemulsions can show different results, probably due to availability of the molecules in emulsive formulations. The preservative PMEPBI had little action in the presence of the excipients tested, but, when incorporated into the formulations, showed antimicrobial activity against the tested microorganisms (Figure 3e,f). Preservatives belonging to the same chemical group are used to produce additive effects when used in combination. This result corroborates the findings of Smaoui and Hlima (2012) who tested PMEPBI in shampoo for children on microorganisms *S. aureus, E. coli, P. aeruginosa, C. albicans,* and *A. niger* and reported efficacy after 14 days of incubation. Paraben combinations have been shown to have a synergistic effect on the bacteria as previously described by Doron *et al.* (2001).

It is important to pay particular attention to excipient and preservative compatibility and preservative concentrations. Cosmetic products containing many ingredients can have a synergistic or antagonistic effect on the effect of preservatives (Steinberg, 2012). The activity of the preservative system should be tested in the product to be preserved because various factors such as composition and processing can influence the preservative action.

CONCLUSIONS

The results demonstrate that preservatives containing parabens are strongly inactivated by non-ionic surfactants. Therefore, paraben-free preservatives are more effective in nanoemulsified formulations because they contain a high content of these surfactants. In addition, a blend of preservative agents may be required, because the preservatives analyzed against nano- and microemulsified formulations were not effective for all microorganisms recommended in the challenge test.

When developing new products with a high surfactant content, it is necessary to know the effectiveness of the preservative system because, although drug incompatibilities are described in the literature, inactivating the potential and the intensity with which they occur also depends on the type of excipient (chemical class) and other components of the formulation, composition of preservative systems, the excipient to preservative ratio, and the availability in the formulation.
CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this paper.

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