INTRODUCTION

In recent years, the number of superficial mycoses (dermatophytosis, candidiasis and geotrichosis) caused by different fungal species in humans and animals has increased considerably. This phenomenon is due to the increase in the number of pathologies included in the context of secondary immunodeficiencies generated by physiological conditions (e.g. age, stress), pathological conditions (e.g. diabetes mellitus, malnutrition), immunosuppressive drugs (e.g. corticoids, chemotherapeutics), and environmental agents (e.g. x-rays, γ, pesticides), among others (Olmo, Alonso de la Espriella, Escobar Sánchez, 2011).

Likewise, the drastic increase in the incidence of fungal infections has been accompanied by an increase in the innate and acquired resistance to antifungal drugs (Pfaller et al, 2011a; Pfaller et al, 2011b; Pfaller et al, 2011c). Therefore, there is a need to search for new therapeutic options against fungal infections. Silver has been used since ancient times to treat infections and remains widely used in a variety of medical applications: treatment of burns, catheter linings, endotracheal tubes, disinfectants and wound dressings (Young, Melaiye, 2005; Kollef et al, 2008). Despite its widespread and continuous use, the relatively few cases of resistance to silver are of paramount importance (Gupta et al, 1999; Li, Nikaido, Williams, 1997; Silver, 2003; Modak et al, 1983; Pirnay et al, 2013).

The aim of the present study was to calculate the Minimum inhibitory concentrations (MICs) of silver nanoparticles and clotrimazole for Candida species and their interaction by the adaptation of standardized methods. The MICs values of clotrimazole were 9 E-04 - 3 E-03 ug/ml, 0.1-0.6 ug/ml, 3 E-03 - 0.1 ug/ml and 3 E-03 - 0.3 ug/ml for Candida albicans susceptible to fluconazole, Candida albicans resistance to fluconazole, Candida krusei and Candida parapsilosis, respectively. The MICs values of silver nanoparticles were 26.50 - 53 ug/ml; 26.50 - 106 ug/ml; 106 - 212 ug/ml and 26.50 - 53 ug/ml for Candida albicans susceptible to fluconazole, Candida albicans resistance to fluconazole, Candida krusei and Candida parapsilosis, respectively. Synergism between clotrimazole and silver nanoparticles was measured by checkerboard BMD (broth microdilution) test and shown only for C. albicans susceptible to fluconazole because the fractional inhibitory concentrations (FICs) values were 0.07 - 0.15 ug/ml. Indifference was shown for the other species tested because the FICs values were between 0.5 – 2 - 3.06 ug/ml. The results suggest synergistic activity depending on the fungus species analysed, however we recommend the incorporation of others measurement methodologies to confirm our results. As for measurement methodologies of MICs of silver nanoparticles and clotrimazole international normative were respected to guarantee reproducible and comparable results.

Keywords: Silver nanoparticles. Antifungal. Clotrimazole. Candida spp.
This lack of silver resistance can be attributed to different suggested mechanisms of action (Rai, Yadav, Gade, 2009). In the case of silver nanoparticles, the broad biocidal effect is due by interrupting phospholipid bilayer of the cell membrane thus increasing its permeability and altering the mechanism of protein synthesis in bacteria (Kalimuthu et al, 2017; Sondi, Salopek-Sondi, 2004; Kasthuri, Kathiravan, Rajendiran, 2009). Moreover, Kim et al. (2009) describe a similar mechanism for antifungal activity of silver nanoparticles by disrupting the structure of the cell membrane and inhibiting the normal budding process on Candida albicans.

It is important to highlight that presently the CIM values data for silver nanoparticles in Candida species and their potentiation studies are rather variable due to their measurement methodology. For example, Hassan et al. (2013) obtained a CIM value of silver nanoparticles for Candida albicans with a non-standardized methodology, thus their results can not be compared with others using another methodology.

Just as Chopra (2007) pointed out the need for silver MIC levels and breakpoints to be developed in bacteria, we point out as well the need to develop and/or evaluate the antifungal capacity of silver with standardized methods.

Likewise, basic research have been carried out for the development of various in vitro techniques (e.g: checkerboard BMD test) that can evidence the interactions between binary mixtures of antimicrobial agents and other biological molecules with antimicrobial activity (Castañeda-Ramírez et al, 2011; Leclercq et al, 2013). The checkerboard BMD test has extensively been used because it is simple and does not require sophisticated mathematical calculations (Jenkins, Schuetz, 2012). To our knowledge there are not registered studies of potentiation of silver nanoparticles with clotrimazole for Candida species with standardized methods.

On the other hand, Clotrimazole is widely used in the treatment of dermatomycosis. It is a member of theazole class of synthetic antimycotic agents that were discovered in the 1960s. The azoles comprise the largest class of antimycotic drugs for clinical use and can be further subdivided into two classes on the basis of their chemical structure: imidazoles and triazoles. Clotrimazole falls into the imidazole subclass of azole drugs. All azole-type antmycotic drugs interfere with the biosynthesis of ergosterol, which is an important component of the fungal cell membrane (Hitchcock et al., 1990). The resultant depletion of ergosterol and its replacement by the aberrant sterol species perturb normal membrane permeability and fluidity (Crowley, Gallagher, 2014).

In this context, the aim of the present study was to calculate the minimum inhibitory concentrations (MIC) of silver nanoparticles and clotrimazole for Candida species and to evaluate whether combined they can enhance their antifungal effect by adapting standardized methods for possible medical and veterinary applications.

**MATERIAL AND METHODS**

**Preparation of silver nanoparticles**

Silver nanoparticles were synthesized by the method described by Vigneshwaran et al, 2006. Briefly, 1.0 g of soluble starch (Biopack) was added to 100 mL of deionized water and heated in a microwave oven (BGH Litton, Generation II, 50 Hz). After complete dissolution, 1 mL of a 100 mM aqueous solution of silver nitrate (Sigma-Aldrich) was added and stirred well. This mixture was kept in an autoclave at 15 psi and 121 °C for 5 min. The resulting solution was light yellow, indicating the formation of silver nanoparticles. The silver nanoparticles were evaluated by the Laboratorio de Microscopía Electrónica, Unidad de Administración Territorial, Centro Científico Tecnológico Conicet-Bahía Blanca, Argentina. The samples were placed on 300-mesh copper plates, coated with Formvar® and observed with a JEOL JSM CIIX Transmission Electron Microscope (TEM), at an acceleration voltage of 80 Kv, with a magnification of 100,000 x. Digitized images were acquired and evaluated with Image Pro Image Analysis and Processing Software.

**Biological material**

The antimycotic activity was tested using four strains of Candida: C. albicans susceptible
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(INM° 982879) and C. albicans resistant (MC°452) to fluconazole from clinical isolates, C. krusei (DMic***134409) and C. parapsilosis (DMic***134410). The strains belong to the Collection of Cultures of Mycology of the Servicio de Antifúngicos, Departamento de Micología, Instituto Nacional de Enfermedades Infecciosas, Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) - Dr. Carlos G. Malbrán, Buenos Aires, Argentina.

Fungicidal assay

The fungicidal activity of clotrimazole and silver nanoparticles was independently evaluated to determine their MICs for the four Candida strains tested.

The MICs values for clotrimazole were evaluated in BMD, based on documents 7.1 and 7.2 of the European Committee for Antimicrobial Susceptibility Tests (EUCAST) - Subcommittee on Antifungal Susceptibility Tests (AFST) (EUCAST-AFST, 2008; Arendrup et al, 2012). For the antifungal evaluation of the silver nanoparticles, this methodology was slightly modified because they had to be in soluble starch. Briefly, half serial dilutions were done in soluble starch starting from a concentration of 100 mM (16987 ug/ml) silver nanoparticles in starch. Each dilution was mixed 1/10 with RPMI 1640 (Sigma-Aldrich) broth. Finally 100 μL of each dilution was mixed with 100 μL of RPMI 1640 broth inoculated with the different Candida species analyzed at a concentration of 1-5 x 10⁵ CFU/mL in the wells of a microtiter plate.

To evaluate the MICs of clotrimazole, half serial dilutions were done in dimethylsulfoxide starting from a concentration of 0.64 ug/ml, each of which was mixed 1/20 with RPMI 1640 broth. Finally 100 μL of each dilution was mixed with 100 μL of RPMI 1640 broth inoculated with the different Candida species analyzed at a concentration of 1-5 x 10⁵ CFU/mL in the wells of a microtiter plate.

The microtiter plates were cultured at 37 °C for 48 h and thereafter read through Cytation 5 Imaging Reader (Biotek) at a wavelength of 530 nm. The MICs values were evaluated in triplicate and taken at the lowest concentration of clotrimazole or silver nanoparticles in which the absorbance was less than or equal to 50% of the absorbance of the growth control.

Evaluation of the binary combination between clotrimazole and silver nanoparticles by checkerboard BMD test

The assay was performed on microtiter plates. Briefly, wells were cultured with the microorganism and the dilutions of clotrimazole and silver nanoparticles to determine susceptibility at a concentration of 10⁶ CFU/mL. The plates were incubated at optimum temperature and time of growth (30-35 °C for 48 h). These dilutions should contain concentrations that include values higher and lower than the MICs previously determined for each agent against the microorganism to be studied. The assay was performed by triplicate. Then, the same assay was performed two more times in separate days, again in triplicate.

By this method, a fractional inhibitory concentration (FIC) was calculated by comparing the MICs of each drug alone with the MICs of that drug in combination. The FIC values were calculated taking into account the minimum and maximum values of the CIMs alone.

Synergy is usually defined as a 4-fold decrease in the MICs of the agents in combination when compared with the agents tested alone (Saiman, 2007). This test was performed for each of the four Candida strains evaluated. The FIC was calculated and interpreted as Jenkins and Schuetz, 2012:

\[ \Sigma \text{FIC} = \text{FIC of agent A} + \text{FIC of agent B} \]

\[ \text{FIC of agent A} = \frac{\text{MIC of agent A in combination}}{\text{MIC of agent A alone}} \]

\[ \text{FIC of agent B} = \frac{\text{MIC of agent B in combination}}{\text{MIC of agent B alone}} \]

Synergy = \( \Sigma \text{FIC} \leq 0.5 \)

Indifference = 0.5 < \( \Sigma \text{FIC} \leq 4 \)

Antagonism = \( \Sigma \text{FIC} > 4 \)

Some researchers consider that compounds are additive when \( \Sigma \text{FIC} \leq 1 \)
RESULTS AND DISCUSSION

Silver nanoparticles

The methodology used for the synthesis of silver nanoparticles generated nanoparticles of homogeneous distribution and size between 5 and 20 nm, with an average of 58 and standard deviation of 65 in the distribution of the silver nanoparticles (Figure 1). These characteristics and the possibility of producing them in soluble starch make this material compatible for both biomedical and pharmaceutical applications.

![Transmission electron microscopy (TEM) micrograph of the silver nanoparticles in soluble starch (100,000 x; JEOL JSM CIIX). b) Size distribution of silver nanoparticles in starch (sample amount= 2 Ml).](image)

MICs values

The MICs values of clotrimazole and silver nanoparticles were shown in Table I. The results observed for *C. albicans* resistant to fluconazole were higher concentrations than the rest of the *Candida* species tested for clotrimazole. However, the concentrations of MICs for silver nanoparticles did not show the same pattern of susceptibility among the different species of *Candida*.
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The chessboard tests between silver nanoparticles and clotrimazole for each *Candida* species are showed in Figure 2. According to the calculations proposed by Jenkins and Schuetz (2012), the interaction between silver nanoparticles and clotrimazole generated the following interpretations in the four *Candida* species. As seen from figure 2, *C. albicans* susceptible to fluconazole decreased more than 4 dilutions the MIC value, while for the other *Candida* species no decrease was observed.

The FICs values for *C. albicans* susceptible to fluconazole were 0.07 μg/ml for the maximum MIC value and 0.15 μg/ml for the minimum MIC value. Both results demonstrate synergism for this combination because, according to Jenkins, when the sum of the FIC of the agents involved in the study generates values below or equal to 0.5 is synergism. However, for *C. albicans* resistant to fluconazole it was 0.5 μg/ml for the maximum value and 2.1 μg/ml for the minimum value, for *C. krusei* it was 0.5 μg/ml for the maximum value and 2 μg/ml for the minimum value and for *C. parapsilosis* it was 0.5 μg/ml for the maximum value and 3.06 μg/ml for the minimum value. According to the FICs values there was indifference in these fungal species for combination of silver nanoparticles with clotrimazole.

**TABLE I - MICs values of clotrimazole and silver nanoparticles**

|                | MICs of clotrimazole (ug/ml) | MICs silver nanoparticles (ug/ml) |
|----------------|-------------------------------|-----------------------------------|
| *C. albicans* (S) | 9.50 E-04, 3.75 E-03 | 26.50-53 |
| *C. albicans* (R) | 0.15-0.62 | 26.50-106 |
| *C. krusei* | 3.75 E-03-0.15 | 106-212 |
| *C. parapsilosis* | 3.75 E-03-0.31 | 26.50-53 |

S: susceptible; R: resistance; MICs: minimum inhibitory concentrations

**Checkerboard BMD test**

The MICs values of clotrimazole in combination with silver nanoparticles and the MICs values of silver nanoparticles in combination with clotrimazole are showed in Table II.

**TABLE II - MICs of clotrimazole in combination with silver nanoparticles and vice versa**

|                | MICs of clotrimazole in combination with silver nanoparticles (ug/ml) | MICs of silver nanoparticles in combination with clotrimazole (ug/ml) |
|----------------|---------------------------------------------------------------------|------------------------------------------------------------------|
| *C. albicans* (S) | 3.02 E-05 | 3.3 |
| *C. albicans* (R) | 1.5 E-02 | 53 |
| *C. krusei* | 3.87 E-03 | 106 |
| *C. parapsilosis* | 7.75 E-03 | 26.5 |

S: susceptible; R: resistance; MICs: minimum inhibitory concentrations
FIGURE 2 - Chessboard tests between silver nanoparticles and clotrimazole for each Candida species analyzed. On the left side (a) the wells highlighted with green of microplate correspond to the absorbances that were greater than 50% of the absorbance of the growth control. On the right side (b) isobologram graphics represented the variation of MICs values caused by the interaction of silver nanoparticles and clotrimazole.
DISCUSSION

The antifungal activity of silver nanoparticles has also been described by Kim et al. (2009), who suggested that the mode of action of silver nanoparticles against fungal pathogens such as Candida species may be by destroying the integrity of the cell membrane and stopping the budding process. Recently, Lee et al. (2019) studied antifungal activity of silver nanoparticles in C. albicans and Saccharomyces cerevisiae, in these work detected an increase of reactive oxygen species (ROS) production after exposure of silver nanoparticles only in C. albicans, but not in Saccharomyces cerevisiae. Therefore, they conclude silver nanoparticles exhibit antifungal activity in a manner that may or may not be ROS dependent, according to the fungal species.

The MICs values of silver nanoparticles obtained in this study showed a less antifungal activity compared to other studies, for example Hassan, Mansour and Mahmoud (2013) obtained values of 2 ug / ml of MIC 50 for silver nanoparticles in Candida albicans. In the case of Juneyoung, Keuk-Jun and Woo Sang (2010) showed antifungal activity of silver nanoparticles against T.mentagrophytes and Candida species in an 80% inhibitory concentration (IC80) range of 1-25 μg / ml. Although the measurement methodologies were not the same, our results of MICs values for silver nanoparticles are much higher. It should be clarified that the comparison of the MICs values in silver nanoparticles for Candida species can be erroneous due to the lack of standardization of the method.

It should be note, even though method was slightly modified because silver nanoparticles must be in soluble starch, in this work respected the rules based on documents 7.1 and 7.2 of the European Committee for Antimicrobial Susceptibility Tests (EUCAST) - Subcommittee on Antifungal Susceptibility Tests (AFST) (EUCAST-AFST, 2008; Arendrup et al, 2012). Therefore, the described methodology could guarantee the reproducibility of MICs results.

As mentioned previously, there are few cases of resistance to silver, our MICs results might suggest that a higher concentration of silver nanoparticles is needed to inhibit Candida resistant to fluconazole and Candida krusei. Bearing in mind that the latter possesses intrinsic resistance to azoles (Sanglard, Odds, 2002), we could infer that the mechanisms of resistance to antifungals are similar to those that generate resistance in silver nanoparticles. Salas-Orozco et al.(2019) suggest that multiresistant antimicrobial bacteria become resistant to silver nanoparticles by the similar molecular mechanisms that generate resistance to antibiotics. In the case of fungicides and silver nanoparticles this “cross-resistance” could be explained the silver nanoparticles CIM results observed of our work, but more studies should be carried out to confirm our assessment.

On the other hand, Aleš Panáček et al. (2009) evaluated the MIC of silver nanoparticles prepared by the modified Tollens process, these nanoparticles were similar in size to those obtained by us in the present study. In this case, recorded the MIC values as the lowest concentration inhibiting the visible growth of microorganisms and found them to be quite low. However, in their methodology of synthesis, they used surfactants with proven cytotoxic activity and that can not be used for biomedical application, reason why not be used in our work.

Sanjenbam, Gopal and Kannabiran (2014) evaluated the antifungal activity of silver nanoparticles synthesized using Streptomyces sp.VITPK1 and despite achieving quite low concentrations of silver nanoparticles against different strains of Candida, the methodology of measurement used was not MIC 50% and thus their results are not comparable to those obtained by us in the present study. However, if a stabilizing agent was added, the methodology used by these authors could also be used for biomedical applications.

Synthesis of nanoparticles using biomaterials (e.g. starch) is simple and more eco-friendly than those synthesized by physical and chemical methods (Bhainsa, D’Souza, 2006). For these reasons, and to obtain silver nanoparticles with distribution and size homogeneous we chose the methodology described by Vigneshwaran et al.(2006). In this methodology, the starch acts as a reducing and stabilizing agent. According to the authors, nanoparticles prepared in this way are found to be stable in solution over a period of three months at room temperature (25 °C) and show no signs of aggregation.
The use of environmentally benign and renewable materials like soluble starch offers numerous benefits of eco-friendliness and compatibility for pharmaceutical and biomedical applications. Moreover, the widespread occurrence of this naturally occurring polysaccharide makes this process amenable to large-scale industrial production.

Regarding the results of the individual MICs for Clotrimazole observed in Table I, the values for Candida albicans susceptible and resistant to fluconazole were the expected ones, because clotrimazole is an antifungal agent of the azoles group, therefore requiring higher concentrations of clotrimazole for inhibiting fluconazole-resistant Candida albicans than for the strain fluconazole-susceptible. These results coincide with the conclusions of Pelletier et al. (2000), who suggest that Candida albicans can develop cross resistance among the azoles group. However, regarding Candida krusei and Candida parapsilopsis, the inhibition pattern was not as expected, since Candida krusei has intrinsic resistance to fluconazole (Pfaller et al. 2008) and Candida parapsilosis is susceptible to it, higher MIC values were expected for the first and more low for the second.

When analyzing other studies reporting clotrimazole MIC values for these Candida species, we can observe that they behaved similarly to the results obtained in our study, for example Hussain Qadri et al. (1986) reported MIC values to Clotrimazole between 0.1 to 4 ug / ml for both C. krusei and C. parapsilosis; Richter et al. (2005) reported MIC values of 0.03 at 0.5 ug / ml for C. parapsilosis and 0.12 at 1 ug / ml for C. Krusei. Both values, the cited by these authors and those our study indicate that in the case of Candida krusei there is no cross resistance between fluconazole and clotrimazole because it has the same inhibition pattern as Candida parapsilosis. When comparing our results with the cited authors we can affirm that the measurement methodology used was correct and a good antifungal sensitivity was obtained.

The results of this study suggest that the silver nanoparticles in the starch showed different antifungal susceptibility patterns than clotrimazole.

In concern to the checkerboard BMD test, in which two antimicrobial agents are serially diluted in a two-dimensional fashion to include all combinations during a specified clinically relevant range, is very useful. This method allows recognizing synergistic, additive, indifferent and antagonistic interactions occurring with the agents being tested. However, the results should be interpreted with caution because they do not take into account pharmacological interactions from a pharmacokinetic or safety perspective for the patient (adverse effect) (Jenkins, Schuetz, 2012).

Tutaj et al. (2016) also evaluated the antifungal activity in a hybrid system between amphotericin and silver nanoparticles. Although they did not measure synergy by the chessboard method, they observed some synergistic activity between amphotericin and silver nanoparticles, mainly against C. albicans. This result coincides with ours for C. albicans susceptible to fluconazole. However, with the others Candida species evaluated in this study, such synergism was not observed.

Regarding the synergistic activity between silver nanoparticles and clotrimazole we concluded that is dependent on the fungal species, since a synergistic effect was observed in C. albicans susceptible to fluconazole because it was the only strain an FIC value lower than 0.5 ug/ml. With respect to C. albicans resistant to fluconazole the slight decrease on the CIM of clotrimazole combined with silver nanoparticles was not enough to consider potentiation because the FIC values of these agents were between 0.5 and 4, which means indifference.

As for C. krusei and C. parapsilosis, the combination of clotrimazole and silver nanoparticles is indifferent with respect to their antifungal activity alone, since also in these strains their FIC values were between 0.5 and 4. Likewise, as can be seen in the Tables I and II the combined MIC values are similar to the lowest values of individual MICs for each one.

As mentioned above, antifungal resistance mechanisms could also generate resistance to silver nanoparticles, so it can be expected that strains wich are more resistant to one or another agent will not generate potentiation in their combination and the strain highly susceptible to both agents generate potentiation in their combination. Riggle, Kumamoto (2000) studied an ATP-dependent copper eflux protein that also mediates the
removal of silver ions in *C. albicans* and was identified as the main component that confers tolerance to silver. It could be inferred that this type of mechanism also participates in antifungal resistance.

In conclusion, more studies need to be carried out on the mechanisms of resistance of silver nanoparticles and their relationship with the mechanisms of resistance to antifungals in *Candida* species to confirm our observations.

While it is important to highlight the incorporation of the chessboard technique to evaluate the interaction between clotrimazole and silver nanoparticles, we must clarify that it could be insufficient for the evaluation of synergism as the only method. To reach more accurate conclusions, measurement methodologies such as time-kill kinetic and / or in vivo evaluations should be incorporated (Jenkins, Schuetz, 2012).

Therefore, although more studies should be conducted with more species of pathogenic fungi and / or others antifungals we consider that this combination should be taken into account for the design of new local medication with antifungal activity due to the synergism observed in *Candida albicans* susceptible to fluconazol and no antagonism in the others species of *Candida* tested.

Finally, the methodology originally proposed by Vigneshwaran et al. (2006) and used in this study generates a silver nanoparticle solution that can be dosed and maintained over time thanks to the starch acting as an stabilizing agent. In this way, solutions and / or creams could be prepared for external applications against different types of mycoses.

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