BJPS

Brazilian Journal of
Pharmaceutical Sciences

http://dx.doi.org/10.1590/s2175-97902022e18719

Article

Antifungal activity of silver nanoparticles and clotrimazole against *Candida* spp.

María Laura Meneses[1,2,3*]; Maite Recalde[2]; Paula Lorena Martin[3]; Alejandro Guillermo Pardo[1]

[1]Laboratorio de Micología Molecular, Instituto de Microbiología Básica y Aplicada, Departamento de Ciencia y Tecnología, Universidad Nacional de Quilmes, Buenos Aires, Argentina, CONICET, [2]Instituto de Ingeniería y Agronomía, Universidad Nacional Arturo Jauretche, Buenos Aires, Argentina, [3]Laboratorio Central, Hospital Escuela, Departamento de Clínicas, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, Buenos Aires, Argentina

The aim of present study was calculate the Minimum inhibitory concentrations (MICs) of silver nanoparticles and clotrimazole for *Candida* species and their interaction by the adaptation of standarized 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.

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*

***Correspondence:** M. L. Meneses. Laboratorio de Micología Molecular. Departamento de Ciencia y Tecnología. Universidad Nacional de Quilmes. Roque Saenz Peña 352, Bernal, Buenos Aires, Argentina, CONICET. Email: meneses.laura@gmail.com

al, 2003). 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 highligth 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 the azole 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 antimycotic 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

(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.
*INM: Instituto Nacional Malbrán; **MC: Micología Clínica; ***Dmic: Departamento de Micología.

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 10^5CFU/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. Seeding in the microtiter plates was performed as described above for silver nanoparticles.

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 10^5 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:

$$\sum FIC = FIC \text{ of agent A} + FIC \text{ of agent B}$$

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

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

$$Synergy = \sum FIC \leq 0.5$$

$$Indifference = 0.5 < \sum FIC \leq 4$$

$$Antagonism = \sum FIC > 4$$

Some researchers consider that compounds are additive when $>0.5 \sum FIC \leq 1$

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.

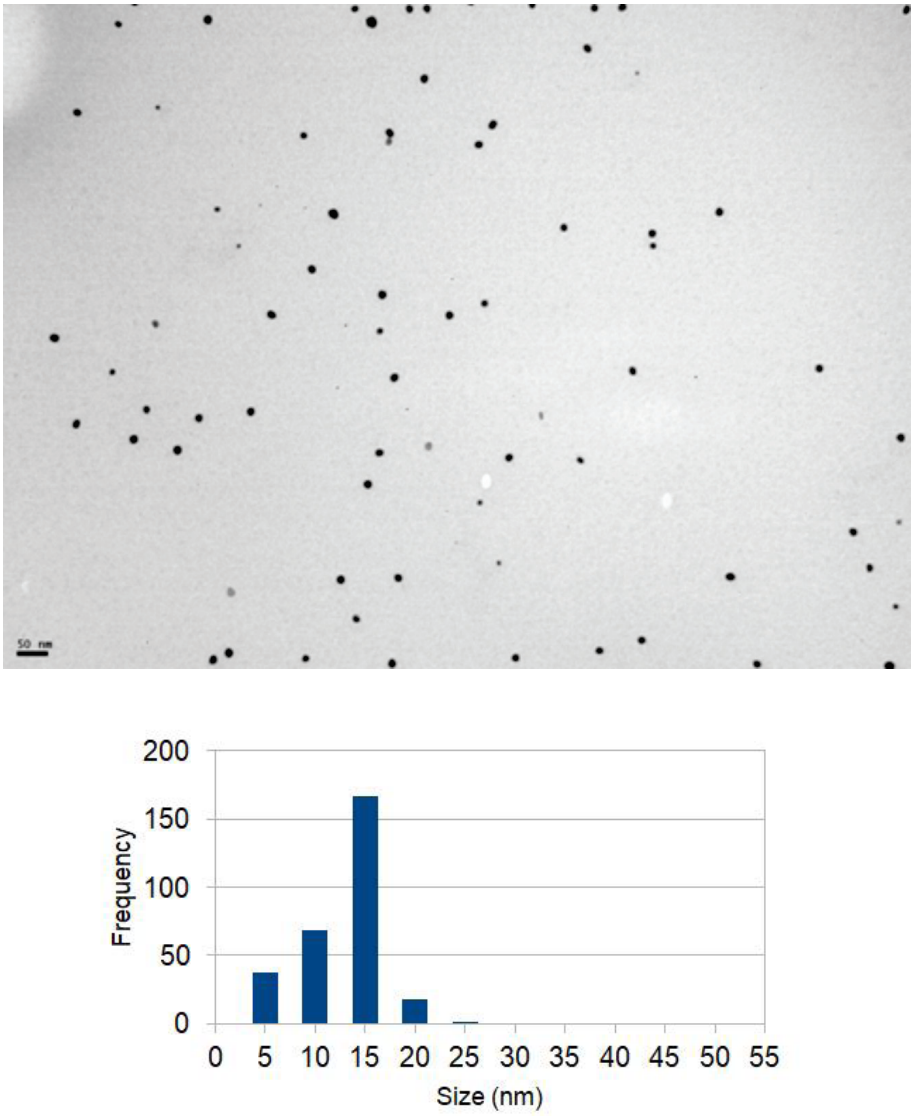

FIGURE 1 - a) Transmission electron microscopy (TEM) micrograph of the silver nanoparticles in soluble starch (100,000 x; JEOL JSM CIIX). b) Size distribution of silver nanoparticles in starch (sample amount= 2 Ml).

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*.

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

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 ug/ml for the maximum MIC value and 0.15 ug/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 ug/ml for the maximum value and 2.1 ug/ml for the minimum value, for *C. krusei* it was 0.5 ug/ml for the maximum value and 2 ug/ml for the the minimum value and for *C. parapsilosis* it was 0.5 ug/ml for the maximum value and 3.06 ug/ml for the minimum value. According to the FICs values there was indifference in these fungal species for combination of silver nanoparticles with clotrimazole.

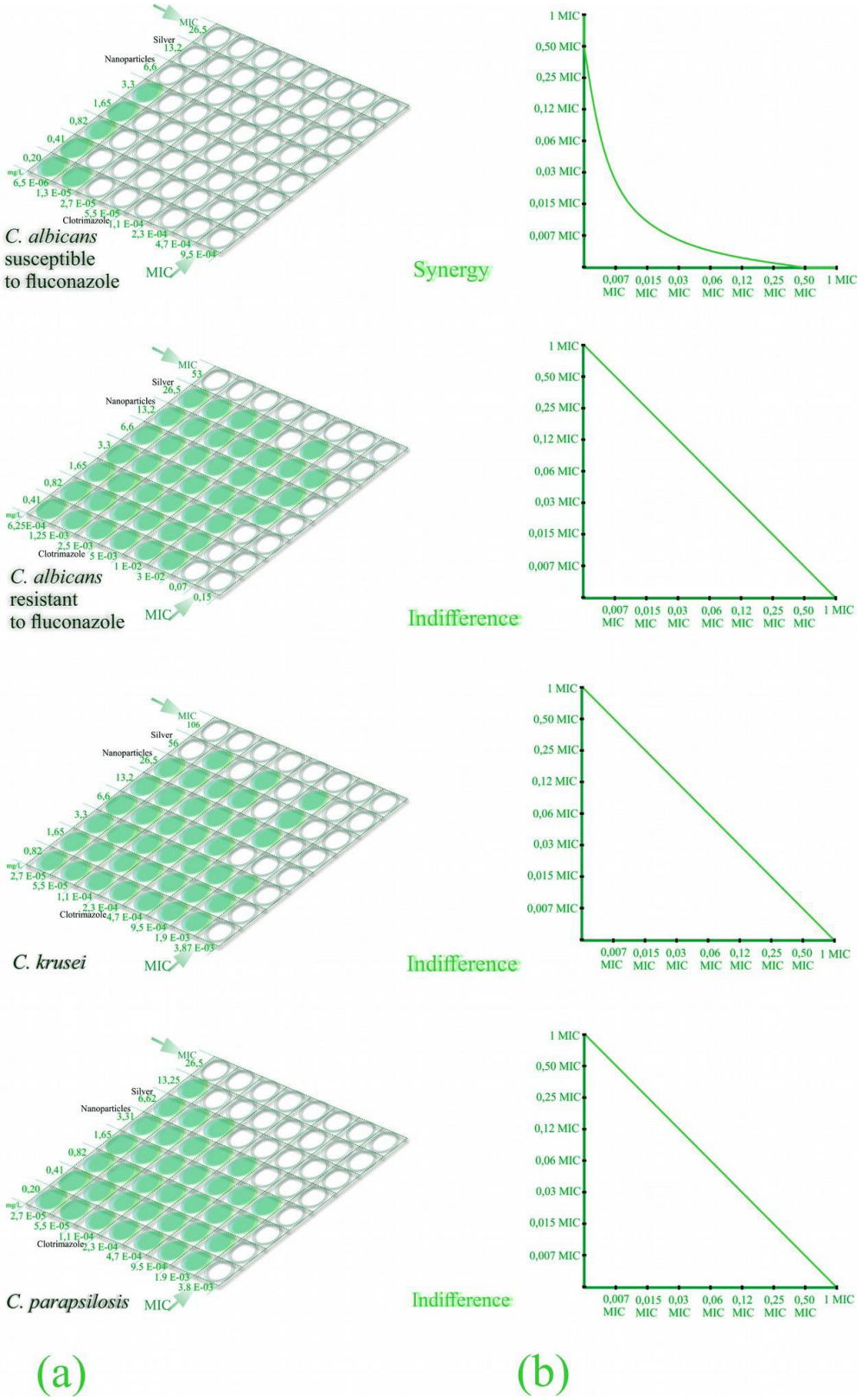

FIGURE 2 - Chessboard tests between silver nanoparticles and clotrimazole for each Candida species analyzed. On the left side (a) the wells highlighted with green of microplate correspond to the absorbances that were greater than 50% of the absorbance of the growth control. On the right side (b) isobologram graphics represented the variaton 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 destructing 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 (IC_{80}) 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 use 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 parapsilopsis* 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. parapsilopsis*; Richter *et al.* (2005) reported MIC values of 0.03 at 0.5 ug / ml for *C. parapsilopsis* 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 parapsilopsis*.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 synergism 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 could 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.

ACKNOWLEDGEMENTS

We would like to thank Dra. Susana Córdoba and Mic. Guillermina Isla of the Servicio de Antifúngicos, Departamento de Micología, Instituto Nacional de Enfermedades Infecciosas (INEI) Administración Nacional de Laboratorios e Institutos de Salud (ANLIS) - Dr. Carlos G. Malbrán, Argentina, for their disinterested support on this research project.This work was funded to M.L.M. by Universidad Nacional Arturo Jauretche, Argentina, project number 301/12**.**

REFERENCES

Arendrup MC, Cuenca-Estrella M, Lass-Flörl C, Hope W. EUCAST-AFST. The European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing. EUCAST DEFINITIVE DOCUMENT EDef 7.2 Revision. Method for the determination of broth dilution minimum Inhibitory concentrations. Clin Microbiol Infect. 2012;18(7):688-689. https://doi.org/10.1111/j.1469-0691.2012.03880.x

Bhainsa KC, D'Souza SF. Extracellular biosynthesis of silver nano-particles using the fungus *Aspergillus fumigatus*. Colloids Surf B. 2006;47(2):160-4.

Castañeda-Ramírez C, Cortes-Rodríguez V, De la Fuente-Salcido N, Bideshi DK, Barboza-Corona JE. Isolation of *Salmonella* spp. from lettuce and evaluation of its susceptibility to novel bacteriocins synthesized by *Bacillus thuringiensis* and antibiotics. J Food Prot. 2011;74(2):274–8.

Chopra I. The increasing use of silver-based products as antimicrobial agents: a useful development or a cause for concern? J Antimicrob Chemother. 2007;59(4):587–590.

Crowley PD, Gallagher HC. Clotrimazole as a pharmaceutical: past, present and future. J Appl Microbiol. 2014;117(3):611-617.

EUCAST-AFST. The European Committee on Antimicrobial Susceptibility Testing-Subcommittee on Antifungal Susceptibility Testing. EUCAST Definitive Document EDef 7.1: method for the determination of broth dilution MICs of antifungal agents for fermentative. Clin Microbiol Infect. 2008;14(4):398–405.

Gupta A, Matsui K, Lo JF, Silver S. Molecular basis for resistance to silver cations in *Salmonella*. Nat Med. 1999;5(2):183–8.

Hassan AA,Mansour MK, Mahmoud HH. Biosynthesis of silver nanoparticles (Ag-Nps) (a model of metals) by *Candida albicans* and its antifungal activity on Some fungal pathogens (*Trichophyton mentagrophytes* and *Candida albicans*). New York Sci J. 2013;6(3):27-34.

Hitchcock AC, Dickinson K, Brown BS, Evans EGV, Adams JD. Interaction of azole antifungal antibiotics with cytochrome *P*-450-dependent 14a-sterol demethylase purified from *Candida albicans*. Biochem J. 1990;266(2):475-480.

Hussain Qadri SMH, Flournoy DJ, Qadri SGM, Ramirez EG. Susceptibility o f clinical isolates o f yeasts to anti-fungal agents. Mycopathologia. 1986;95(3):183-187.

Jenkins SG, Schuetz AN. Current concepts in laboratory testing to guide antimicrobial therapy. Mayo Clin Proc. 2012;87(3):290–308.

Juneyoung L, Keuk-Jun K, Woo Sang S, Jong Guk K and Dong Gun L. The Silver Nanoparticle (Nano-Ag): a New Model for Antifungal Agents, Silver Nanoparticles. 2010. ISBN: 978-953-307-028-5, *InTech*. http://www.intechopen.com/books/silver-nanoparticles/the-silver-nanoparticle-nano-ag-a-new-model-for-antifungal-agents

Kalimuthu K, Panneerselvam C, Chou C, Li-Chun T, Murugan K, Kun-Hsien T, et al. Control of dengue and *Zika* virus vector *Aedes aegypti* using the predatory copepod *Megacyclops formosanus*: synergy with *Hedychium coronarium*-synthesized silver nanoparticles and related histological changes in targeted mosquitoes. Process Saf Environ Prot. 2017;109:82–96.

Kasthuri J, Kathiravan K, Rajendiran N. Phyllanthin-assisted biosynthesis of silver and gold nanoparticles: a novel biological approach. J Nanopart Res. 2009;11(5):1075–1085.

Kim KJ, Sung WS, Suh BK, Moon SK, Choi JS, Kim JG, et al. Antifungal activity and mode of action of silver nano-particles on *Candida albicans*. Biometals. 2009;22(2):235–42.

Kollef MH, Afessa B, Anzueto A, Veremakis C, Kerr KM, Margolis BD, *et al*. Silver-coated endotracheal tubes and incidence of ventilator-associated pneumonia. NASCENT Investigation Group. The NASCENT randomized trial. JAMA. 2008;300(7):805–13.

Lee B,Lee MJ, Yun SJ, Kim K, Choi IH, Park S. Silver nanoparticles induce reactive oxygen species-mediated cell cycle delay and synergistic cytotoxicity with 3-bromopyruvate in *Candida albicans*, but not in *Saccharomyces cerevisiae*. Int J Nanomed. 2019:14:4801–4816.

Leclercq R, Cantón R, Brown DF, Giske CG, Heisig P, MacGowan AP, et al. EUCAST expert rules in antimicrobial susceptibility testing. Clin Microbiol Infect. 2013;19(2):141–60.

Li XZ, Nikaido H, Williams KE. Silver-resistant mutants of *Escherichia coli* display active efflux of Ag+ and are deficient in porins. J Bacteriol. 1997;179:6127–32.

Modak SM, Stanford JW, Bradshaw W, Fox CL Jr. Silver sulfadiazine (AgSD) resistant *Pseudomonas* infection in experimental burn wounds. Panminerva Med. 1983;25(3):181–8.

Olmo C, Alonso de la Espriella G, Escobar Sánchez L. Curso continuo de actualización en Pediatría CCAP. 2011;11(1).

Panáček A, Kolář M, Večeřová R, Prucek R, Soukupová J, Kryštof V, et al. Antifungal activity of silver nanoparticles against *Candida* spp. Biomaterials. 2009;30(31):6333–6340.

Pelletier R, Peter J, Antin C, Gonzalez C, Wood L, Walsh TJ. Emergence of resistance of *Candida albicans* to clotrimazole in human immunodeficiency virus-infected children: in vitro and clinical correlations. J Clin Microbiol. 2000;38(4):1563–1568.

Pfaller MA, Diekema DL, Gibbs VA, Newell E, Nagy S, Dobiasova, et al. *Candida krusei*, a Multidrug-Resistant Opportunistic Fungal Pathogen:Geographic and Temporal Trends from the ARTEMIS DISK Antifungal Surveillance Program, 2001 to 2005. J Clin Microbiol. 2008;46(2):515–521.

Pfaller MA, Moet GJ, Messer SA, Jones RN, Castanheira M. *Candida* bloodstream infections: comparison of species distributions and antifungal resistance patterns in community-onset and nosocomial isolates in the SENTRY Antimicrobial Surveillance Program, 2008-2009. Antimicrob Agents Chemother. 2011a;55(2):561–6.

Pfaller MA, Moet GJ, Messer SA, Jones RN, Castanheira M. *Candida* bloodstream infections: comparison of species distribution and resistance to chinocandin and azole antifungal agents in Intensive Care Unit (ICU) and non-ICU settings in the SENTRY Antimicrobial Surveillance Program(2008-2009). Int J Antimicrob Agents. 2011b;38(1):65–9.

Pfaller MA, Moet GJ, Messer SA, Jones RN, Castanheira M. Geographic variations in species distribution and echinocandin and azole antifungal resistance rates among *Candida* bloodstream infection isolates: Report from the SENTRY Antimicrobial Surveillance Program (2008 to 2009). J Clin Microbiol. 2011c;49(1):396–9.

Pirnay JP, De Vos D, Cochez C, Bilocq F, Pirson J, Struelens M, et al. Molecular epidemiology of *Pseudomonas aeruginosa* colonization in a burn unit: persistence of a multidrug-resistant clone and a silver sulfadiazine-resistant clone. J Clin Microbiol. 2003;41(3):1192–202.

Rai M, Yadav A, Gade A. Silver nanoparticles as a new generation of antimicrobials. Biotechnol Adv. 2009;27(1):76–83.

Richter SS, Galask RP, Messer SA, Hollis RJ, Diekema DJ, Pfaller MA. Antifungal Susceptibilities of *Candida* Species Causing Vulvovaginitis and Epidemiology of Recurrent Cases. J Clin Microbiol. 2005;43(5):2155–2162.

Riggle PJ0, Kumamoto CA. Role of a *Candida albicans* P1-Type ATPase in resistance to copper and silver ion yoxicity. J Bacteriol. 2000;182(17):4899–4905.

Saiman L. Clinical utility of synergy testing for multidrugresistant *Pseudomonas aeruginosa* isolated from patients with cystic fibrosis: "the motion for." Paediatr Respir Rev. 2007;8(3):249–55.

Salas-Orozco M, Niño-Martínez N,Martínez-Castañón GA, Torres Méndez F, Compean Jasso ME, Ruiz F. Mechanisms of resistance to silver nanoparticles in endodontic bacteria: a literature review. J Nanomater. 2019; Article ID 7630316, https://doi.org/10.1155/2019/7630316.

Sanglard D, Odds FC. Resistance of *Candida* species to antifungal agents: molecular mechanisms and clinical consequences. Lancet Infect Dis. 2002;2(2):73–85.

Sanjenbam P, Gopal JV, Kannabiran K. Anticandidal activity of silver nanoparticles synthesized using *Streptomyces* sp.VITPK1. J Mycol Med. 2014;24(3):211–9.

Silver S. Bacterial silver resistance: molecular biology and uses and misuses of silver compounds. FEMS Microbiol Rev. 2003;27(2-3):341–53.

Sondi I, Salopek-Sondi B. Silver nanoparticles as antimicrobial agent: a case study on *E. coli* as a model for Gram-negative bacteria. J Colloid Interface Sci. 2004;275(1):177–182.

Tutaj K, Szlazak R, Szalapata K, Starzyk J, Luchowski R, Grudzinski W, et al. Amphotericin B-silver hybrid nanoparticles: synthesis, properties and antifungal activity. Nanomedicine: Nanotechnology. Biol Med. 2016;12(4):1095–1103.

Vigneshwaran N, Nachane RP, Balasubramanya RH, Varadarajan PV. A novel one-pot "green" synthesis of stable silver nanoparticles using soluble starch. Carbohydr Res. 2006;341(12):2012–8.

Young WJ, Melaiye A. Silver and its application as an antimicrobial agent. Expert Opin Ther Pat. 2005;15(2):125–30.

Received for publication on 07th September 2018
Accepted for publication on 12th September 2019