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Comparison of Two Green Technologies for the Growth Control of Methicillin-Resistant *Staphylococcus aureus*

Comparación de dos tecnologías verdes para el control del crecimiento de *Staphylococcus aureus* resistente a la meticilina

Raquel A. Villamizar Gallardo[1]*, Karla Cruz Cuellar[1], Edgar Emir González Jiménez[2] y Johann F. Osma[3]

[1] Department of Microbiology, School of Basic Sciences. Universidad de Pamplona. Km 1, vía Bucaramanga, ciudadela Universitaria. Edifice Eduardo Cote EC 202, 2do. Piso. Pamplona, Colombia.

[2] Geophysics Institute, School of Engineering. Pontificia Universidad Javeriana. Carrera 28 A No. 39A-63. Bogota, Colombia.

[3] CMUA. Department of Electrical and Electronic Engineering. Universidad de los Andes. Cra 1 Este, No. 19A-40. Bogotá, Colombia. Correspondence: raqvillamizar@unipamplona.edu.co

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RESUMEN

Introducción. El Staphylococcus aureus resistente a la meticilina (MRSA) es una bacteria patógena que según la Organización Mundial de la Salud (OMS) debería incluirse en la lista de patógenos prioritarios frente a los que se deberían desarrollar nuevos mecanismos de tratamiento, dada su resistencia a los antibióticos. Objetivo: En este estudio se comparó el efecto bactericida de dos productos obtenidos por química verde como lo son los aceites esenciales y las nanopartículas de plata, contra una cepa de MRSA aislada de alimentos. Métodos. La evaluación de la inhibición de la bacteria se basó en pruebas de difusión en disco y microdilución en placa de pocillos en medios de cultivo base modificados con tres tipos diferentes de

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aceites esenciales y dos tipos de nanopartículas de plata como agentes bactericidas. Además, se utilizó microscopía electrónica de barrido (SEM) para visualizar el daño a nivel estructural de las bacterias. Resultados: Los resultados permitieron determinar que el aceite esencial Lippia origanoides exhibió mayor eficacia en la inhibición del crecimiento de MRSA, con una concentración mínima inhibitoria y una concentración mínima bactericida de 2 mg/ml. Por el contrario, las nanopartículas de plata mostraron poco efecto significativo en la reducción y/o inhibición del crecimiento del patógeno estudiado. Microscópicamente, fue posible observar la pérdida total de la integridad de la pared celular de las bacterias. Conclusiones. Se pudo determinar que los aceites esenciales fueron más eficientes que las nanopartículas de plata en la inhibición de MRSA y, por lo tanto, podrían usarse como una alternativa verde efectiva para controlar el crecimiento de bacterias patógenas en matrices alimentarias.

Correspondence Raquel A. Villamizar Gallardo[1]*: raqvillamizar@unipamplona.edu.co

Palabras clave: Agentes antibacterianos, nanopartículas, plata, aceites volátiles.

ABSTRACT

Introduction. Methicillin-resistant *Staphylococcus aureus* (MRSA) is a pathogenic bacterium that according to the World Health Organization (WHO) should be included in the list of priority pathogens towards which new treatment mechanisms should be developed, given its resistance to antibiotics. **Objective:** In this study, the

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bactericidal effect of two products obtained by green chemistry such as essential oils and silver nanoparticles, were compared against a MRSA strain isolated from food. **Methods.** The evaluation of the inhibition of the bacteria was based on disk diffusion tests and well plate microdilution in base culture media modified with three different types of essential oils and two types of silver nanoparticles as bactericidal agents. In addition, scanning electron microscopy (SEM) was used to visualize damage at the structural level of the bacteria. **Results:** The results allowed determining that the essential oil *Lippia origanoides* exhibited greater efficacy in inhibiting the growth of MRSA, with a minimum inhibitory concentration and a minimum bactericidal concentration of 2 mg / ml. On the contrary, silver nanoparticles showed little significant effect in reducing and / or inhibiting the growth of the studied pathogen. Microscopically, it was possible to observe total loss of the bacteria cell wall integrity. **Conclusions.** It was possible to determine that essential oils were more efficient than silver nanoparticles inhibiting MRSA and, therefore, could be used as a green effective alternative to control pathogenic bacteria growth in food matrices.

Keywords: Antibacterial agents, nanoparticles, silver, volatile oils.

INTRODUCTION

Staphylococcus aureus is a bacterial strain that normally habitats mucous and skin from humans and animals. However, this strain can colonize the human body due to virulent factors such as enzymes, cytotoxins, exotoxins, exfoliative toxins, among others, that ease its access to any organ or tissue, generating suppuration, tissue necrosis, vascular thrombosis and bacteremia (Gómez, et al., 2016; Oliveira, et al., 2018). The World Health Organization has recently published the list of priority pathogens to focus the research and development of new antibiotics (WHO-World Health Organization', 2019) and Methicillin-Resistant *Staphylococcus aureus* (MRSA) was included on the high priority category with indication of medium sensibility and resistance to vancomycin. Its resistance to methicillin has been associated to the presence of a mobile genetic element known as SCCmec where gen mecA is included. This encodes a penicillin binding protein, PBP2a, that enables transpeptidase activity in the presence of β-lactams (Naghshbandi, 2018), and hence the ability to restore or biogenesis of the cell wall (Lahiri S, Alm R., 2016).

MRSA has been recognized as a serious public health threat worldwide, causing more than 80,000 life-threatening infections per year in the US (CDC, 2019). The European Centre for Disease Prevention and Control reported in 2018, more than 43000 isolated strain of *S. aureus* from different sources, from which more than 13% belonged to the group methicillin resistant (ECDC, 2019). Currently, there are four types of infections caused by MRSA strains: Hospital acquired or HA-MRSA (Lakhundi S, 2018), Community acquired or CA-MRSA (Scientific Report of EFSA and ECDC.EU, 2019), Livestock-associated MRSA or LAMRSA (Mascaro, 2019) and Food Borne Associated-MRSA or FBA-MRSA (Herrera et al.,2016; Can et al., 2017). This classification reveals the adaptive capacity of this bacteria to different microenvironments (Basanisi et al., 2017). A study conducted in Colombia, showed that *S. aureus* was identified in meat products such as ground beef and pork chop meat from 40 outlets located at three locations in the city of Cartagena. The results obtained revealed the presence of the mecA gene in 88% of the analyzed samples (López et al., 2017). Therefore, there is a need for developing effective but also eco-friendly methods, focused towards the control of multidrug resistant bacteria.

Some promising alternatives include the use of essential oils and silver nanoparticles (AgNP). Essential oils have shown to be highly effective in the growth inhibition of multiresistant bacteria (György et al., 2020). These hydrophobic compounds can adhere to the membrane of bacterial cells, affecting their pressure, increasing their permeability, causing the leakage of ions present inside the cell (Chouhan, et al., 2017). AgNP are colloidal dispersions within the range of 10 to100 nm with antibacterial effect, due to its surface to volume ratio, that allows them easily penetrate through porins present in the bacterial cell structure, reach the DNA and corrupt the cellular replication process, inhibit the electron transport chain, causing the cell death (Villamizar, 2016). The biocidal effect of silver nanoparticles had been tested in at least 12 species of bacteria, including multiresistant bacteria (Barros, et al., 2018; Faizan, et al., 2019).

In the present study, the antimicrobial effect of two different green technologies against a FB-MRSA strain was evaluated, in order to find viable and easy-to-apply strategies to control multidrug bacterial growth, especially in the food industry. Three essential oils, *Lippia origanoides, Lippia alba* and *Eucalyptus globulus*, were analyzed as part of the first technology; while pristine silver nanoparticles (P-AgNP) and P-AgNP functionalized with silanes with an amine terminal group, were tested as the second green technology. An antibiotic of known bactericidal spectrum was used as control for all experiments.

MATERIALS AND METHODS

Microorganisms: MRSA isolated from artisan double cream cheese and genotypically characterized through molecular methods (Herrera, et al., 2016), was provided by professor Fanny Herrera from the Department of Microbiology of the University of Pamplona. *Escherichia coli* (ATCC25922) and *Staphylococcus aureus* (ATCC6538), from the collection of the Universidad de Pamplona (Colombia), were used as control strains.

Essential oils-The essential oils of *Lippia origanoides* (LO), *Lippia alba* (LA) and *Eucalyptus globulus* (EG) were provided by Promotora de Innovación en Biotecnología S.A.S (Promitec) (Santander, Colombia).

Cultures-Microorganisms were cultured in Soy Tripticase Broth (TSB) under 150 rpm agitation at 37 °C for 18 h. Stocks, prepared from these cultures, were adjusted to a 0.5 McFarland concentration (1.5 x 10^8 CFU / mL).

Synthesis, characterization and functionalization of silver Nanoparticles-Pristine silver nanoparticles (P-AgNP) were synthesized using 5 mM $AgNO_3$ (Sigma Aldrich, USA) as the metal salt precursor, and ascorbic acid and citric acid (Faizan, et al., 2019), obtained from Tahiti lime extract (*Citrus latifolia Tan*), as reducing and stabilizing agents. The extract was prepared by filtering the fruit juice with Whatman paper, heated to boiling temperature for 5 minutes and centrifuged at 8,000 rpm for 15 minutes to remove impurities. A percentage of citric acid equals to 4.8 ± 0.4% was determine by titration with sodium hydroxide N 0.095 and 1% phenolphthalein, while 0.4 ± 0.1% of ascorbic acid concentration was determined by titration with iodine. A 1:3 ratio (vol:vol) of silver salt solution and extract reacted for 5 h at 25 °C to obtain nanoparticles (P-AgNP) as shown in Figure 1A. The colloidal sample was washed thoroughly by centrifugation and then characterized by UV-Vis spectrophotometry and scanning electron microscopy (SEM). The absorbance spectra were obtained using a UV-Vis UV-2600 spectrophotometer (Shimadzu, Japan) in the range from 300 to 800 nm. SEM characterization was carried out using a Zeiss Evo HD 15 SEM (Carl Zeiss AG, Germany) to determine morphology, distribution and size of the obtained nanoparticles. The functionalization of P-AgNP with silanes was carried out by adjusting the pH of the containing solution to 8.0 with NaOH, and then sonicated for 20 min using a Branson M2800 (Branson Ultrasonic, USA). 1 mL of 2.7 M Tetramethylammonium hydroxide (TMAH) (Sigma Aldrich, USA) per gram of P-AgNP was added to the mixture and sonicated for 10 min to ease the further silanization process. 2 µL of 3-aminopropyltriethoxysilane (APTES) (Sigma Aldrich, USA) was added and the mixture was sonicated for 10 min. APTES functionalized P-AgNP were coded as A-AgNP.

Evaluation of inhibition agents-The inhibitory effect of the three essential oils (LO, LA and EG)[22] and the two types of

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pristine silver nanoparticles (P-AgNP and A-AgNP) was evaluated. As a control method, antibiogram using gentamicin was carried out due to the resistance to cefoxitin, oxacillin, penicillin, and ampicillin of the MRSA strain. Inhibitory effect was evaluated on all bacterial strains by disc diffusion test and well microdilution test.

Disk diffusion Test-Disk diffusion tests were carried by triplicate out using Petri dishes (60 x 15 mm) with TSA and adding 100 µL of each strain at a 0.5 McFarland concentration (1.5 x 10^8 UFC/mL). 6 mm semi-disks, impregnated with 20 µL of an essential oil or a type of nanoparticle, were placed in the Petri dishes and incubated at 37 °C. The bacterial growth around the semi-disks was continuously monitored from 24 to 96 h, by measuring the inhibition halos with a caliper. The levels of susceptibility and/or resistance were evaluated according to the Clinical and Laboratory Standards Institute (CLSI) standard inhibition halos established for *S. aureus* and *E. coli* against different groups of antibiotics according to the diameter of the evaluated area as: Sensible (S) ≥15 mm, Intermedium (I) 12-15 mm and Resistant (R) ≤12 (CLSI,, 2015).

Determination of the Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC)-The MIC and MBC were determined by using broth dilution and agar plug diffusion methods as reported elsewhere Balouiri M et al, with some modifications (Balouiri et al., 2016). Briefly, 25 µL of bacterial suspension was added at a McFarland 0.5 concentration to each well. Then, 50 µL of TSB and 10 µL of Trifenyl Tetrazolium Chloride (CTT) (Sigma Aldrich, USA) and 25 µL of different concentrations of the inhibition agent was added (e.g. EOs and AgNPs). Positive controls were prepared with bacterial suspension, antibiotic and CTT. In the case of the negative control, TSB plus CTT was added. Plates were incubated at 37 °C for 24 h. The MIC was the lowest concentration at which cell viability was not observed (color change) after 24 h of incubation. After that, 100 µL from the MIC was placed into Petri dishes with TSA and incubated for 24 hours at 37 °C, in order to determine the MBC. The MBC was calculated for the selected essential oils, as the lowest concentration in which no apparent growth was observed in the plate, compared to the positive control. All assays were conducted by triplicate.

SEM characterization of antibacterial effect-MRSA samples before and after being exposed to essential oils were observed using a Zeiss EVO HD 15 SEM. Samples were dried until critical point, then coated with gold and analyzed in low vacuum, to evidence the inhibitory effect of the microbicidal agents.

RESULTS

Synthesis, characterization and functionalization of silver nanoparticles-The green synthesis of P-AgNP produced nanoparticles with sufficient monodispersity and structural quality for the experimentation. The VIS spectra showed a resonance peak at 430 nm (Figure 1A) consistent with the presence of AgNP of a size between 30 and 40 nm in diameter. The microphotographs obtained by SEM (Figure 1B) confirmed that the mean values of the P-AgNP as 36 ± 9 nm. The particle size distribution obtained (Figure 1C) is characteristic of colloids synthesized by green chemistry, due to the use of citric and ascorbic acid. P-AgNP showed an anionic behavior with a measured zeta potential of -20 mV.

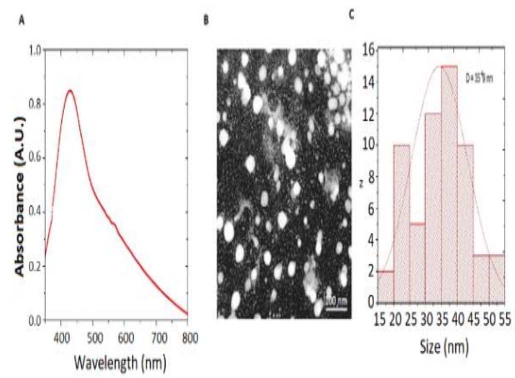

Figure 1. (**a**) VIS spectra of P-AgNP; (**b**) SEM Microphotography of P-AgNP; (**c**) Size distribution of P-AgNP.

Inhibition tests carried out using P-AgNP and A-AgNP showed no inhibitory effect on MRSA *or S. aureus* (Figure 2A), only an intermediate sensitivity was detected on *E. coli* when applying A-AgNP compared to the gentamicin control. By contrast, the inhibitory capacity of different essential oils on Gram-positive and Gram-negative pathogens showed that LO was the essential oil with the greatest antibacterial effect on all strains, being

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highly effective on the MRSA strain. LA was also effective in inhibiting MRSA and *S. aureus*; however, *E. coli* was resistant to it. EG was not effective in inhibiting any of the three analyzed strains (Figure 2B).

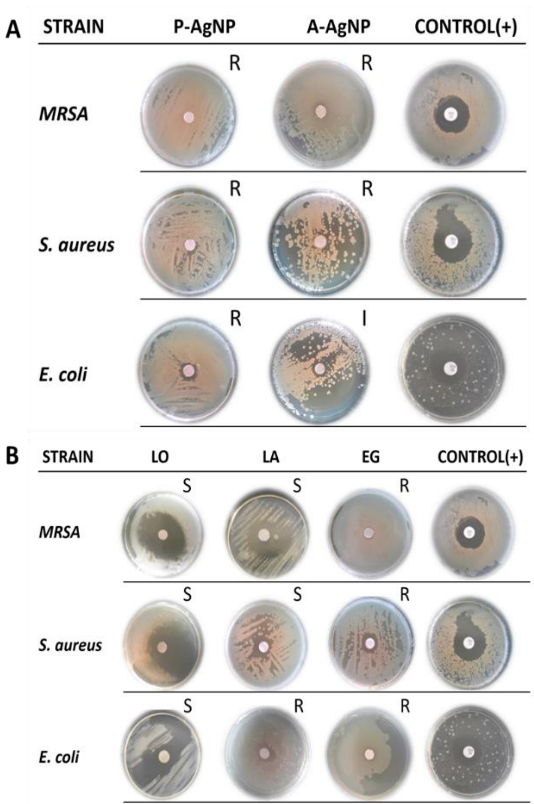

Figure 2. Inhibitory effect of A) Pristine silver nanoparticles (P-AgNP) and APTES functionalized silver nanoparticles (A-AgNP) and B) Essential oils (LO, LA, EG), against MRSA, *S. aureus* and *E. coli*. (S: Sensible/I: Intermedium / R: Resistant).

The average inhibition zones of each essential oil can be seen in relation to each strain are shown in figure 3.

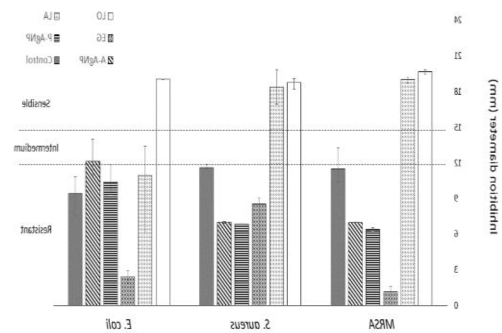

Figure 3. Susceptibility and resistance shown by the three bacterial strains against each of the three essential oils and two types of nanoparticles tested

Determination of the Minimum Inhibitory Concentration (MIC) and minimum bactericidal concentration (MBC)-The minimum inhibitory concentration (MIC) and the minimum bactericidal concentration (MBC) was calculated only for the essential oils LO and LA, due to their high bactericidal effect. Table 1 shows the MIC results for the tested essential oils.

Table 1. MIC of the essential oils *L. origanoides* (LO) and *L. alba* (LA) against MRSA, *S. aureus* and *E. coli*.

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[LO] (mg/ml)	MRSA	*S. aureus*	*E. coli*	[LA] (mg/m)	MRSA	*S. aureus*	*E.*
6,0	–	–	–	10,0	–	–	–
4,0	–	+	–	8,0	–	–	–
2,0	–	+	–	6,0	–	+	–
0,8	+	+	+	4,0	+	+	–
0,6	+	+	+	2,0	+	+	+
0,4	+	+	+	0,8	+	+	+
0,2	+	+	+	0,6	+	+	+
0,1	+	+	+	0,4	+	+	+
0,08	+	+	+	0,2	+	+	+

The MBC determined by agar plug diffusion for LO was 2 mg/ml, against MRSA and *E. coli*, respectively. As for *S. aureus*, the MBC of LO was determined as 6 mg/ml, showing a 3-yield to achieve an inhibition of 99.9% of the bacteria present. In the case of LA, the MBC for the MRSA was 6 mg/ml; for *S. aureus* 8 mg/ml and for *E. coli* 4 mg/ml.

SEM analysis showed in detail the effect of the essential oil LO on the bacterial cell. Figure 4A shows the untreated MRSA, while in Figure 4B it can be observed how LO caused a complete loss of the cell wall integrity and therefore, leading to cell lysis.

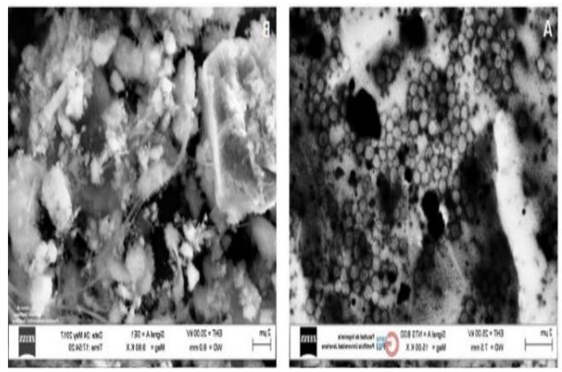

Figure 4. (**A**) SEM image of untreated MRSA strain (**B**): SEM image of MRSA strain after being exposed to 2 mg/ml of *L. origanoides* (LO).

DISCUSSION

Synthesis of silver nanoparticles was consistent with those reported by Manikprab *et al, 2016*. AgNP mechanism action is based on its ability to penetrate through the cellular structure (wall and/or cell membrane) and access the ultrastructure breaking through the processes of cellular respiration, synthesis of nucleic acids, altering the cell wall of the bacteria and causing bacterial lysis. In the present study it was possible to determine that the effect of the pristine (P-AgNP) and surface modified silver nanoparticles (A-AgNP) was reduced against MRSA and *S. aureus* strains. These are Gram-positive bacteria with a thick peptidoglycan layer which makes it

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difficult for the silver nanoparticles to access the cytoplasm and therefore, their microbicide action is inhibited. In addition, bacterial resistance against nanomaterials especially, AgNP has been previously referenced in other studies and is linked to genetic alterations in some bacterial strains (Xuan, et al., 2018). However, this particular aspect must be studied in detail in further researches. In the case of *E. coli*, a weak effect of A-AgNP was observed. This agrees with Cavassin and collaborators, who showed that AgNP modified with citrate and chitosan were highly effective in inhibiting the growth of antibiotic resistant pathogens (Cavassin, et al., 2015). This indicates that functional groups coupled after the nanoparticles biosynthesis displayed a critical role on the interaction with bacterial cellular receptors. APTES is a positively charged molecule which reacts by means of electrostatic interactions with the negatively charged groups present on the bacterial cell surface. This effect of receptor-ligand interaction could be slightly appreciated against *E. coli*, where the negatively charged lipopolysaccharides (LPS) present in its outer membrane could have reacted with the amino groups present in the APTES molecules and thus, facilitating the transport of the AgNP to the inner cell structure. Although the A-AgNP bactericide effect against *E. coli* was higher than those obtained with MRSA, it was lower than those observed with the control gentamicin.

By contrast, the essential oils LO and LA were efficient in the inhibition of both Gram negative and Gram positive bacteria. Similar results were reported by Medeiros and collaborators, whose remarking that carvacol (37.3%), thymol (22.4%) and ´Y-Terpinene (10.9%) were the main active principles of the *L. origanoids* and could be used in a synergy way with aminoglycosides against illnesses caused by MRSA (Medeiros, et al., 2014). These chemical compounds are highly lipophilic, with high affinity for the plasmatic membrane, causing the loss of its integrity; increasing their permeability mainly to K+ and H+ ions and producing loss of cytoplasmic content, dissipation of the proton motive force, lysis and cell death (Man, et al., 2019). Regarding to the antibacterial effect of *L. alba*, Porfirio E and collaborators found a 100 % of inhibition of *S. aureus* biofilm cells using a range of 1 and 2 mg/mL of this essential oils (Porfirio et al., 2017). In the present study, it was found that concentrations between 4 mg/mL and 8 mg/mL of *L. alba,* were necessary to inhibit both, the Gram negative and Gram positive strains. Finally, the EG oil showed no inhibitory effect against *S. aureus, MRSA and E. coli*, respectively, indicating the need to increase the concentration of this essential oil in order to achieve significant inhibition of bacterial growth. Similar results were obtained by Maya J and collaborators, who´s recently published that EG exhibited a weak inhibitory effect against clinical isolates of the methicillin resistant and methicillin sensitive *Staphylococcus aureus* (Maya et al., 2019).

CONCLUSIONS

It was established that the antimicrobial effect of essential oils, was greater than modified and pristine silver nanoparticles, against all tested bacteria. Within the group of the essential oils, it is important to highlight the bactericidal activity of *Lippia origanoides*. With a MBC of 2 mg/mL, this essential oil was capable of inhibiting the bacterial growth of both, Gram positive and Gram negative bacteria, including MRSA. Therefore, it could be an efficient and ecofriendly compound, able to control resistant and no resistant pathogens in different food matrices.

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Conflict of interest statement: Authors declare not having any conflict of interest around the work reported in the present paper.

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