ANTIBACTERIAL ACIVITY OF GOLD AND SILVER NANOPARTICLES IMPREGNATED WITH ANTIMICROBIAL AGENTS

Geovana Dagostim Savi

Mestrado em Ciências da Saúde pela Universidade do Extremo Sul Catarinense – UNESC, Criciúma, SC; Doutoranda do Programa de Pós-Graduação em Ciências dos Alimentos pela Universidade Federal de Santa Catarina – UFSC, Florianópolis, SC; E-mail: geovanasavi@gmail.com.

Ana Claudia Trombin

Laboratório de Microbiologia Experimental, Programa de Pós-Graduação em Ciências da Saúde, Unidade Acadêmica de Ciências da Saúde, Universidade do Extremo Sul Catarinense – UNESC, Criciúma, SC.

Jaqueline da Silva Generoso

Mestre em Ciências da Saúde pelo Programa de Pós Graduação em Ciências da Saúde (PPGCS) da Universidade do Extremo Sul Catarinense – UNESC, Criciúma, SC; Doutoranda do Programa de Pós Graduação em Ciências da Saúde (PPGCS) da Universidade do Extremo Sul Catarinense – UNESC, Criciúma, SC.

Tatiana Barichello

Doutora em Ciências Biológicas pela Universidade Federal do Rio Grande do Sul – UFRGS, Porto Alegre, RS; Docente no Programa de Pós-graduação em Ciências da Saúde da Universidade do Extremo Sul Catarinense – UNESC, Criciúma, SC.

Jonathann Correa Possato

Graduando em Farmácia, na Universidade do Extremo Sul Catarinense – UNESC, Criciúma, SC; Bolsista de Iniciação Científica.

João Vitor Vieira Ronconi

Graduando em Engenharia Química pela Universidade do Extremo Sul Catarinense – UNESC, Criciúma, SC; Bolsista de Iniciação Científica.

Marcos Marques da Silva Paula

Doutor em Ciência e Engenharia de Materiais pela Universidade Federal de Santa Catarina – UFSC, Florianópolis, SC; Docente titular da Universidade do Extremo Sul Catarinense – UNESC, Criciúma, SC.

ABSTRACT: Introduction: The bactericidal activity of Ag-NPs and Au-NPs impregnated with ceftriaxone, rifampicin and vancomycin is evalauted. **Results:** Ag-NPs impregnated with rifampicin, vancomycin and ceftriaxone showed a respective inhibition zone of 15.0 mm, 11.3 mm and 26.0 mm to *E.coli*. Whereas Au-NPs impregnated with rifampicin and vancomycin showed a non inhibition zone, ceftriaxone showed a zone of 27.0 mm. When Ag-NPs were impregnated with rifampicin, vancomycin and ceftriaxone, they showed a zone of 10.0 mm against *P. aeruginosa*, but Au-NPs showed a non inhibition zone. When Ag-NPs were impregnated against *S. aureus* with rifampicin, vancomycin and ceftriaxone, they showed an inhibition zone of 36.7 mm, 18.0 mm and 10.0 mm, respectively. When Au-NPs were impregnated with vancomycin, rifampicin and ceftriaxone, they showed an inhibition zone respectively of 36.0 mm, 18.0 mm and 13.3 mm. **Conclusion:** Results showed that the efficiency of the nanoparticles impregnated with antibiotics may depend on the antibiotics used.

KEY WORDS: Nanoparticles; Antimicrobial Agents; Synergism; Microorganisms.

ATIVIDADE ANTIBACTERIANA DE NANOPARTÍCULAS DE OURO E PRATA IMPREGNADAS COM ANTIMICROBIANOS

RESUMO: Introdução: No presente estudo, nós avaliamos a atividade bactericida das Ag-NPs e Au-NPs impregnadas com rifampicina, ceftriaxona e vancomicina. **Resultados:** As Ag-NPs impregnadas com rifampicina frente a *E.coli*, demonstraram uma zona de inibição de 15 mm, com vancomicina apresentaram 11,3 mm e com ceftriaxona 26 mm. Quando as Au-NPs foram impregnadas com rifampicina e vancomicina não demostraram zona de inibição, no entanto, a ceftriaxona demostrou 27 mm. Frente à *P. aeruginosa*, as Ag-NPs impregnadas com vancomicina, rifampicina e ceftriaxona apresentaram 10 mm, no entanto, as Au-NPs não demostraram zona de inibição. Frente à *S. aureus*, as Ag-NPs impregnados com rifampicina apresentaram zona de inibição de 36,7 mm, com vancomicina apresentaram 18 mm e com ceftriaxona 10 mm. Quando as Au-NPs foram impregnadas com vancomicina mostraram uma zona de inibição de 36 mm, com rifampicina apresentaram 18 mm e com ceftriaxona 13,3 mm. **Conclusão:** Nós observamos que a eficiência das nanopartículas impregnadas com os antimicrobianos pode depender do antimicrobiano utilizado.

PALAVRAS-CHAVE: Antimicrobianos; Micro-organismos; Nanopartículas; Sinergismo.

INTRODUCTION

Nanotechnology is emerging as a rapidly growing field with applications in Science and Technology for the manufacture of new materials at the nanoscale level (ALBRECHT; EVAN; RASTON, 2006). 'Nano' is a Greek word synonymous to 'dwarf' or 'extremely small' (GONG; LI; HE, 2007). Nanoparticles are clusters of atoms with sizes ranging between 1–100 nm, whilst a 'nano' is used to indicate one billionth of a meter (BRIGGER; DUBERNET; COUVREUR, 2002; RAI; YADAV; GADE, 2009). The burgeoning new field of nanotechnology, opened up by rapid advances in science and technology, is creating myriads of new opportunities for advancing medical science and disease treatment in human health care (SAHOO; PARVEEN; PANDA, 2007).

Metal nanoparticles (Me-NPs) with a high specific surface area and a high fraction of surface atoms have been studied extensively because of their unique physicochemical characteristics which include catalytic activity, optical properties, electronic properties, antimicrobial activity and magnetic properties (KOWSHIK; ASHTAPUTRE; KHARRAZI, 2003; DURAN et al., 2005). Metals have been used for centuries as bactericidal and bacteriostatic agents, among which may be mentioned silver, gold and zinc, each with different properties and activity spectra (PHAN et al., 2004; LANSDOWN, 2006). The antibacterial metals activity depends on their contact surface; a nanoparticle´s larger surface area allows a broader gamut of interactions with other organic and inorganic molecules (HOLISTER et al., 2003). In fact, silver or gold nanoparticles have been used extensively in many bactericidal fields (FU; VARY; LIN, 2005; MELAIYE et al., 2005) and their predominant antimicrobial activity may be attributed to their strong cytotoxicity against several bacterial cells. In fact, they can interact with the functional groups on the bacterial cell surface and inactivate bacteria (YOUNG; LEINWEBER; THOMAS, 2005; RAY et al., 2007; FENG et al., 2000).

Silver nanoparticles (Ag-NPs) have been known to have inhibitory and bactericidal effects. It may be expected that the high specific surface area and high fraction of surface atoms of Ag-NPs will lead to high antimicrobial activity when compared with bulk silver metal (CHO et al., 2005). The silver broad antimicrobial effect is well known and it has been used in different medical fields for years, either for wound healing or in biomaterials. *In vitro* assessment studies on the antibacterial properties and cytotoxicity of nanoparticles silver in bone cement combined good antibacterial activity with an absence of cytotoxicity (ALT et al., 2004).

Gold nanoparticles (Au-NPs) have one of the most important applications in the medical field due to the fact that they are readily taken up by cells (CHITRANI; GHAZANI; CHAN, 2006). The toxicity of Au-NPs against different cell types depends on their size (PAL; TAK; SONG, 2007). Au-NPs bonding to macromolecules has been exploited in many encapsulation processes of materials of clinical interest and Au-NPs have been used to detect antigens in conjugation with antibodies (NAM; THAXTON; MIRKIN, 2003). The conjugation of Au-NPs with biological important molecules, such as oligosaccharides, DNA and proteins, has recently made a great impetus (TATON; MIRKIN; LETSINGER, 2000).

The ability of pathogenic bacteria in resisting antimicrobial agents is a phenomenon which has emerged in recent years and constitutes a major health problem. Although antimicrobial agents may be used against a large number of pathogenic bacteria, many have already developed resistance through genetic mutations (LEEB, 2004; NORRBY; NORD; FINCH, 2005; MORONES et al., 2005). Nanoparticles impregnated with antimicrobial agents may therefore be advantageous: synergism may occur between them which will lead towards an increasing antibacterial activity. Antimicrobial agents have many active groups that react with nanoparticles and microorganisms are unlikely to develop resistance against silver or gold nanoparticles when compared to antimicrobials (AHMAD; SHAHVERDI; FAKHIMI, 2007).

It is therefore important to study the effect of nanoparticles impregnated with antimicrobial agents because they may be used with lower antimicrobial concentrations and because of the increased bacterial resistance of antimicrobials. Current study evaluates the bactericidal activity of silver and gold nanoparticles impregnated with ceftriaxone, rifampicin and vancomycin against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa*.

2 METHODS

2.1 PREPARATION OF AG-NPS

$AgNO_3$ was weighed and dissolved in ultra-pure water and placed in a round bottom flask submerged in a bath of iced water (temperature about 0 °C); sodium citrate was weighed and dissolved in ultra-pure water and the procedure was the same as that with nitrate silver; after adding citrate solution, it was stirred for a few minutes; sodium borohydride was dissolved in ultra-pure water. One aliquot of the solution was retrieved and diluted in ultra-pure water under vigorous stirring and added to an aliquot of the diluted solution of borohydride in a round-bottom flask. The solution changed to a yellow crystalline liquid. SPR showed an UV-visible band with λ_{max} at 430 nm, typical of nanoparticles (SONAVANE; TOMODA; MAKINO, 2008).

2.2 PREPARATION OF AU-NPS

Sodium citrate was weighed and dissolved in ultra-pure water and aurochlorhydric acid was diluted in ultra-pure water. Aurochlorhydric acid was placed in a flask and heated at average temperature; when temperature rose to 90 °C, it was turned off and added to an aliquot of dilute citrate and heated to maximum temperature for 20 minutes. SPR showed an UV-visible band with λ_{max} at 520 nm indicating nanoparticles (TURKEVICH; STEVENSON; HILLIER, 1951).

2.3 IMPREGNATION OF NANOPARTICLES WITH ANTIMICROBIAL AGENTS

Rifampicin was weighed and dissolved with distilled water (1mg/100mL) and ceftriaxone and vancomycin were weighed and dissolved with distilled water (1mg/16 mL). Further, 500 μL of rifampicin 5μg, ceftriaxone 30μg and vancomycin 30 μg were retrieved and impregnated with 500 μL of silver or gold nanoparticles, separately. The pure antimicrobial agents were diluted in 500 μL of distilled water.

2.4 ANTIMICROBIAL ACTIVITY

The antimicrobial activity of the materials was evaluated by the well method, as standardized by Clinical and Laboratory Standards Institute (CLSI), with modifications. The bacteria *Staphylococcus aureus* (ATCC 6538), *Pseudomonas aeruginosa* (ATCC 27853) and *Escherichia coli* (ATCC 25922) were inoculated on Mueller Hinton, Cetrimide and MacConkey media, respectively. The cultures were kept in the media at 37 °C for 24 h. After incubation one sample of each culture was diluted with a saline solution (NaCl 0.9%) up to 0.5 McFarland standard to obtain a bacterial cell density around 10^8 CFU/mL. One aliquot of this suspension was spread on the medium (Mueller–Hinton) of each plate and distributed homogeneously. Wells were performed with 6 mm in diameter in the culture medium and were subsequently distributed aseptically inside wells, in triplicate. Further, 60μL of silver and gold nanoparticles impregnated with rifampicin, vancomycin and ceftriaxone were also deposited inside the wells; control comprised only the antimicrobial agent and silver and gold nanoparticles. All plates were incubated at 37 °C for 24 h. After incubation, the presence of bacterial growth inhibition zone around the samples was observed and the diameter in millimeters was measured (CLSI, 2009; SANDOVAL et al., 2008; YILDIRIM et al., 2007; KURTARAN et al., 2005).

3 RESULTS

Fig. 1 shows a single band with maximum absorption at 520 nm that corresponds to the Surface Plasmon Resonance (SPR) band characteristic of spherical gold nanoparticles.

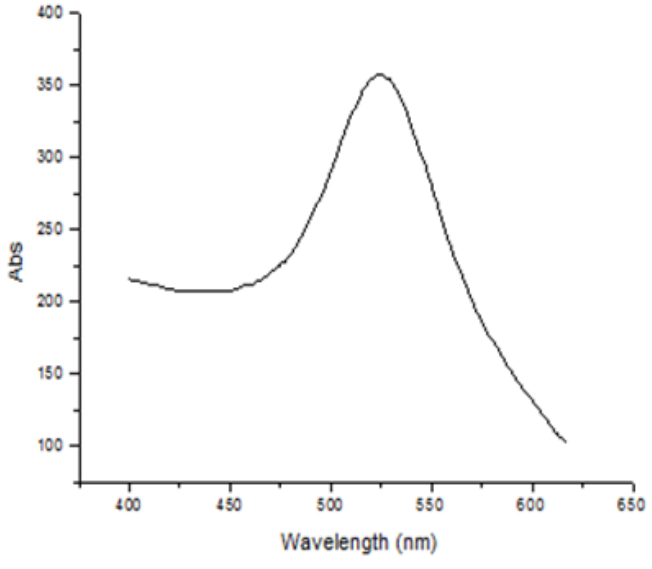

Figure 1. Electronic spectrum for nanogold aqueous solution, showing Surface Plasmon Resonance (SPR).

Fig. 2 shows Surface Plasmon Resonance (SPR) absorption spectrum band for nanoparticles aqueous emulsion. It reveals a single band with maximum absorption at 430 nm that corresponds to the Surface Plasmon Resonance (SPR) band, characteristic of spherical silver nanoparticles.

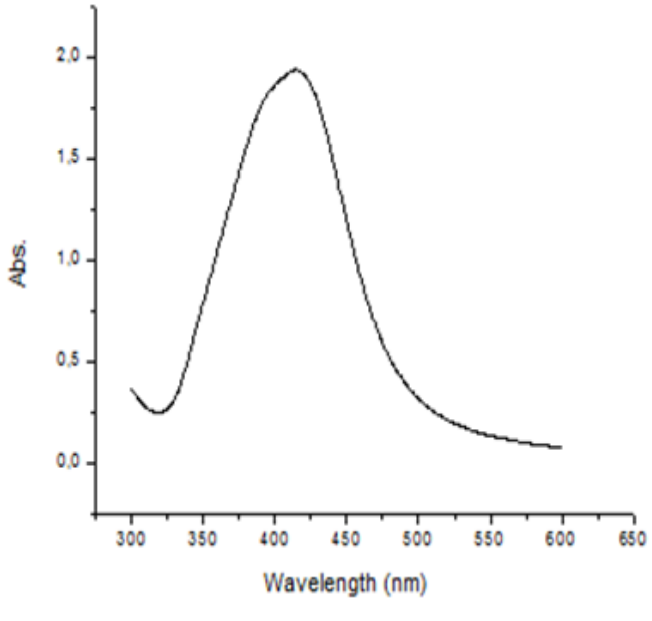

Figure 2. Electronic spectrum for nanosilver aqueous solution, showing Surface Plasmon Resonance (SPR).

Rifampicin showed no inhibition zone against *E. coli*; Ag-NPs impregnated with the antimicrobial agent showed an inhibition zone of 15.0 mm (± 0), but rifampicin with Au-NPs showed no inhibition zone. Rifampicin showed no inhibition zone against *P. aeruginosa*; Ag-NPs impregnated with the antimicrobial agent showed inhibition zone of 10.0 mm (± 0), and rifampicin with Au-NPs showed no inhibition zone. Rifampicin showed an inhibition zone of 33.7 mm (± 1.15) against *S. aureus*; Ag-NPs impregnated with the antimicrobial agent showed inhibition zone of 36.7 mm (± 1.15) and rifampicin with Au-NPs showed an inhibition zone of 36.0 mm (± 1) (Table 1).

Table 1. Antimicrobial activity of silver and gold nanoparticles impregnated with rifampicin

Antimicrobial and nanoparticles	Inhibition zone in mm (mean ± standard deviation)		
	E. coli	*P. aeruginosa*	*S. aureus*
Rifampicin (control)	0	0	33,7 (±1,15)
Rifampicin and silver nanoparticles	15(±0)	10(±0)	36,7(±1,15)
Rifampicin and gold nanoparticles	0	0	36 (±1)
Silver nanoparticles	10(±0)	15(±0)	15(±0)
Gold nanoparticles	0	0	0

Ceftriaxone showed an inhibition zone of 27.0 mm (± 0) against *E.coli*; Ag-NPs impregnated with this antimicrobial agent showed an inhibition zone of 26.0 mm (± 0) and ceftriaxone with Au-NPs showed an inhibition zone of 27.0 mm (± 0). Ceftriaxone showed no inhibition zone against *P. aeruginosa*; Ag-NPs impregnated with the antimicrobial agent showed an inhibition zone of 10.0 mm (± 0) and ceftriaxone with Au-NPs showed no inhibition zone. Ceftriaxone showed an inhibition zone of 12.6 mm (± 0.6) against *S. aureus*; ceftriaxone with Ag-NPs showed an inhibition zone of 10.0 mm (± 0) and ceftriaxone with Au-NPs showed an inhibition zone of 13.3 mm (± 1.15) (Table 2).

Table 2. Antimicrobial activity of silver and gold nanoparticles impregnated with ceftriaxone

Antimicrobial and nanoparticles	Inhibition zone in mm (mean ± standard deviation)		
	E. coli	*P. aeruginosa*	*S. aureus*
Ceftriaxone (control)	27(±0)	0	12,6 (±0,6)
Ceftriaxone and silver nanoparticles	26(±0)	10(±0)	10(±0)
Ceftriaxone and gold nanoparticles	27(±0)	0	13,3 (±1,15)
Silver nanoparticles	10(±0)	15(±0)	15(±0)
Gold nanoparticles	0	0	0

Vancomycin showed no inhibition zone against *E. Coli*; Ag-NPs with the impregnated antimicrobial agent showed an inhibition zone of 11.3 mm (± 1.15) and vancomycin with Au-NPs showed no inhibition zone. Vancomycin showed no inhibition zone against *P. aeruginosa*; Ag-NPs with impregnated antimicrobial agent showed an inhibition zone of 10.0 mm (± 0); vancomycin with Au-NPs showed no inhibition zone. Vancomycin showed an inhibition zone of 18.0 mm (± 0) against *S.aureus*; Ag-NPs with impregnated antimicrobial agent showed inhibition zone of 18.0 mm (± 0) and vancomycin with Au-NPs showed an inhibition zone of 18.0 mm (± 0) (Table 3).

Table 3. Antimicrobial activity of silver and gold nanoparticles impregnated with vancomycin.

Antimicrobial and nanoparticles	Inhibition zone in mm (mean ± standard deviation)		
	E. coli	*P. aeruginosa*	*S. aureus*
Vancomycin (control)	0	0	18 (±0)
Vancomycin and silver nanoparticles	11,3(±1,15)	10(±0)	18(±0)
Vancomycin and gold nanoparticles	0	0	18 (±0)
Silver nanoparticles	10(±0)	15(±0)	15(±0)
Gold nanoparticles	0	0	0

Ag-NPs against *E. coli* showed an inhibition zone of 10.0 mm (± 0); Ag-NPs against *P. aeruginosa* which showed an inhibition zone of 15 mm (± 0); and Ag-NPs against *S. aureus* showed an inhibition zone of 15.0 mm (± 0). Au-NPs showed no inhibition zone against *E. coli*, *P. aeruginosa* and *S. aureus*.

4 DISCUSSION

It is known that Ag-NPs have strong antimicrobial effects, and many researchers are interested in using other inorganic nanoparticles as antibacterial agents, especially since silver in minute concentrations is non-toxic to humans (CRABTREE et al., 2003; FURNO et al., 2004; ABUSKHUNA et al., 2004; HAMOUDA et al., 2000). Conjugates of Au-NPs with antibiotics and antibodies have also been used for selective photothermal killing of protozoa and bacteria. These inorganic nanoparticles have a relevant advantage over conventional chemical antimicrobial agents. Generally, the chemical agents´ antimicrobial mechanism depends on the specific binding with the agents´ surface and metabolism in the microorganism. In fact, several microorganisms have developed drug resistance over many generations. With the increase in microbial resistance against several antibiotics many researchers have tried to develop new and effective antimicrobial agents free of any decreased resistance (PISSUWAN et al., 2010; HUANG; TSAI; CHEN, 2007; ZHAROV et al., 2006).

Grace and Padian (2007) demonstrated that Au-NPs alone did not have any microbial activity. It only acted as a carrier for these antibiotics. Au was important because its large surface area allowed it to carry a large number of antibiotics. In current study, it was verified that antibiotics capped with Au-NPs were effective against various bacteria strains when compared with pure antibiotics. This fact suggested that the presence of Au-NPs did not have any adverse or side effects on the microbial activities, whereas a positive effect was only exerted over the system, i.e. while carrying the antibiotics, say for antibiotic delivery, there was no negative effect imparted on the system due to the presence of gold.

Willis et al. (2006) showed that Au-NPs themselves did not affect bacterial growth or functional activity. In another study, it was reported that there were no significant differences in antibacterial activity between pure gentamicin and its mixture with Au-NPs (BURYGIN et al., 2009). Current results demonstrated the same effect, or rather, Au-NPs showed no inhibition zone against the tested microorganisms. However, when impregnated with antibiotics ceftriaxone and vancomycin, the antimicrobial activity was not altered. In current study, when

Au-NPs were impregnated with rifampicin against *S. aureus*, there was a increase in the inhibition zone. According to studies by Grace and Padian (2007), Au-NPs possess a large surface to volume ratio; because of this large surface area, more antibiotic molecules get adsorbed on gold surfaces. The gold particle surrounded by a number of antibiotic moieties now acts as a single group against the microorganisms. These studies verified that Au-NPs acted as an effective carrier or anchor to these antibiotics.

Ag-NPs have also been studied due to their inhibitory and bactericidal effects. Silver antimicrobial mechanisms may include modifications of sulfhydryl-containing biomolecules such as proteins, the electrochemical collapse gradients across the bacterial cell membranes and the generation of reactive oxygen species (MAILLARD; DENYER, 2006; SHRIVASTAVA et al., 2007). It is believed that heavy metals release ions that react to thiol or sulfhydryl groups (-SH) of proteins on the surface. These proteins protrude through the bacterial cell membrane allowing the entry of nutrients; silver ions (Ag+) replace the hydrogen cation (H+) of the sulfhydryl group that inactivates the proteins by decreasing the permeability that may lead to cell death. The silver nanoparticles under analysis exhibited excellent antibacterial activity against bacteria *S.aureus, Bacillus cereus, E.coli* and *P.aeruginosa* (FENG et al., 2000; CLEMENT; JARRET, 1994). The same occurred in current results which showed that Ag-NPs formed a zone of inhibition against *E.coli, P.aeruginosa* and *S.aureus.*

Ahmad, Shahverdi and Fakhimi (2007) found that the highest increase in area in the presence of Ag-NPs was observed for vancomycin, amoxicillin and penicillin G against *S. aureus*. The effects of Ag-NPs against the antibacterial activity of the antibiotics for *E. coli* were lower than that in *S. aureus*. In another study, it was verified that the antibacterial activities of ampicillin, kanamycin, erythromycin and chloramphenicol were increased in the presence of Ag-NPs against *S.aureus* and *E.coli.* There was a less significant effect on growth of the inhibition zone on gram-positive bacteria, such as *S. aureus*, than on gram-negative bacteria, such as *E.coli* (FAYAZ et al., 2010). In current studies, there were no changes in vancomycin antimicrobial activity when impregnated with silver nanoparticles against *S.aureus*. The antimicrobial activity was increased when rifampicin was impregnated with Ag-NPs for *E.coli*. The synergism was probably caused by the binding reaction between antibiotic and silver nanoparticles, since antibiotic molecules exhibited groups such as hydroxyl and starch groups that may react easily with silver nanoparticles. Therefore, in addition to its antimicrobial activity, the silver nanoparticles probably functioned as an antibiotic carrier (SAHA et al., 2007).

Vancomycin and ceftriaxone impregnated with silver nanoparticles and gold showed no increase of inhibition zone against the *S.aureus*, *E.coli* and *P.aeruginosa* when compared with the antibiotic alone, according to methodology applied in current study. In current investigation, the silver nanoparticles impregnated with vancomycin, rifampicin and ceftriaxone decreased the inhibition zone when compared with Ag-Nps alone against *P. aeruginosa*. The above probably occurred because of high microorganism resistance to the antibiotic. There was not synergism between antimicrobial and nanoparticles, which may have diluted the antimicrobial concentration in the nanoparticles presence and reduced the antimicrobial effect.

Current study reported different results, depending on the antibiotic tested. Only rifampicin occurred synergistically with silver and gold nanoparticle, increasing the inhibition zone against *S.aureus* and only with silver nanoparticle increasing the inhibition zone against *E.coli*. According to Grace and Padiam (2007), depending on the antibiotic used, increase in the activity of the antibiotic-colloidal-gold mixture ranged from 12 to 40%, when compared to the native antibiotic activities. Antibacterial antibiotics activities were enhanced by Au-NPs. Studies demonstrated that, depending on the antibiotic used, the antibiotics antibacterial activities may be improved with Au-NPs or Ag-NPs (GRACE; PADIAM, 2007; SELVARAJ; ALAGAR, 2007; LI et al., 2005).

5 CONCLUSION

According to the reviewed studies and to data from current study, it was observed that the nanoparticles´ efficiency, impregnated with antibiotics, may depend on the antibiotic used. Since synergism was not

observed in most, its activity did not increase against the tested microorganisms. Only rifampicin with silver and gold nanoparticles showed favorable results in the methodology used in this study. Further studies are important to verify the activity of nanoparticles impregnated with other antimicrobials to investigate the synergistic behavior and the consequent improvement of activity of the antibiotics.

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