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RESEARCH ARTICLE

The antibacterial potential of biosynthesized silver nanoparticles using beech bark and spruce bark extracts

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Introduction: Lately, nanotechnology focuses on the green synthesis of AgNPs, using different plant materials, as this method is accessible, cost-efficient, and ecological. The study aimed to investigate the antibacterial potential of AgNPs synthesized using beech/spruce bark extracts (BBE/SBE) and silver salts (acetate/nitrate). **Method**: The growth rates of *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922 were evaluated in the presence of the AgNPs solutions. The checkerboard method was performed to evaluate if these solutions exert synergistic activity with gentamicin. **Results**: For *E. coli*, synergistic effects were observed for the combination of gentamicin 0.25mg/L with AgNP BBE Nit (0.145mg/mL) and with AgNP SBE Ac (0,09mg/mL). For *S. aureus*, no synergistic effects were observed. Overall, the AgNP BBEs solutions combined with gentamicin presented lowest values of fractional inhibitory concentration than the ones registered for the combination of AgNP SBEs with gentamicin, for both bacterial strains. The growth rate of *S. aureus* was inhibited by all the tested AgNPs at the measured time points. For *E. coli*, after 24 hours of incubation, the growth rate was inhibited only in the presence of AgNP SBE Ac. After 6 hours of incubation, the growth rate of *E. coli* was almost stationary in the presence of AgNP BBE Nit. **Conclusions**: The biosynthesis of AgNPs is a valuable choice for obtaining substances with antibacterial potential.

Keywords: silver nanoparticles, beech bark, spruce bark, antibacterial potential

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Introduction

Nowadays, the emerging incidence of the infections caused by multidrug-resistant microorganisms is a major problem for physicians around the world, leading to high rates of morbidity and mortality, prolonged hospitalization, increased costs, and decreased treatments efficacity [1]. It is recognized that, among other factors, the extensive usage of antibiotics is an important cause for the multidrug-resistant bacterial strains widespread. There are a few well-described mechanisms for bacterial resistance, including the production of enzymes such as β-lactamases or aminoglycosides, the production of efflux pumps, decreasing the membrane permeability, modifying the target site of the antibiotics, cell adaptability [2–4]. Because of the antibiotic resistance, in the last decades, the researchers were compelled to study and describe new antimicrobial agents, that might be used as adjuvants or even as treatment options. Nanoparticles are considered an interesting therapeutic alternative, as this solutions act by direct contact with the bacterial wall, avoiding the antibiotic resistance mechanisms developed by the bacteria [4]. Besides investigating the antimicrobial activity of different nanoparticles, some studies also focused on identifying their antimicrobial mechanisms, if and how they can interfere with biofilms production, bacterial adherence, etc. Along with the therapeutic potential of these substances, other applications might be taken into consideration. For example, they can be used in preventing the contamination of different medical devices (orthopedics, orthodontics, suture devices, catheters, etc.) [5–7].

Among the different types of nanoparticles, metal nanoparticles showed a broad spectrum of antimicrobial activity, against both Gram-positive and Gram-negative bacteria [8–10]. There is a special interest for the study of silver nanoparticles (AgNPs), as silver presents important antibacterial activity, well known even from the pre-antibiotic era, along with anti-inflammatory and antioxidant activity [1,11,12]. Another important fact about silver is that, for developing resistance against it, one bacterial generation needs to undergo three separate mutations in three different bacterial systems, a situation that is unlikely to appear [13]. The AgNPs present a higher antimicrobial activity compared to the silver, because of their increased surface/volume ratios, chemical reactivity, increased solubility [10,13]. Even if the mechanisms of the antibacterial activity of the AgNPs are still not completely described, it was proven that these nanoparticles can damage the bacterial cells by disrupting the peptidoglycan, the cytoplasm membrane, the proteins, the DNA, and by generating oxygen reactive species. Sometimes these mechanisms can be combined [4,8,13,14].

Lately, nanotechnology focuses on the green synthesis of the AgNPs, using different plant materials (leaves, bark,

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fruits, flowers, rhizome, etc.), as this is an available, easy, cost-efficient, ecological alternative for the classic synthesis (physical or chemical). These methods of biosynthesis provides AgNPs with controlled size and shape, without other contaminants, making these solutions even more advantageous [15–18]. The plant extracts provides an important source of oils, enzymes, flavonoids, terpenoids, phenolic compounds, that can reduce the silver ions to form the AgNPs and also, acts as stabilizing agents [19–22]. Along with the active involvement in the AgNPs synthesis, the plant extract alone can provide important antibacterial activity, by their high content of alkaloids, tannins, terpenoids, phenolic compounds, with proven antimicrobial activity [23,24].

In recent studies, AgNPs synthesized with beech bark and spruce bark extracts were characterized, and their antibacterial activity was demonstrated [21,22,25]. This study aimed to further investigate the antibacterial activity of these AgNPs, by evaluating their influence on the growth rate of Gram-positive/Gram-negative bacterial strains and to evaluate if these solutions exert synergistic activity with gentamicin, a broad-spectrum antibiotic agent.

Materials and Methods

Bacterial strains

For this study, a Gram-positive bacterial strain (*Staphylococcus aureus* ATCC 25923) and Gram-negative bacterial strain (*Escherichia coli* ATCC 25922) were used. These bacterial strains, part of the collection from the Microbiology Laboratory UMPhST ("George Emil Palade" University of Medicine, Pharmacy, Science, and Technology of Târgu Mureş), were stored at -70°C, and revitalized before each experiment on Columbia blood agar (Oxoid, United Kingdom).

The synthesis of the AgNPs

The protocols used for the preparation of the beech bark (*Fagus sylvatica* L.) extract, the spruce bark (*Picea abies* L.) extract, and the AgNPs were previously published [21,25].

Briefly, 10 g of beech/spruce bark extract were mixed with distilled water (100 mL), placed in an ultrasonic water bath for 30 minutes, and heated at 70°C. Afterward, the obtained extracts were filtered and filled with distilled water (to obtain a final volume of 100 mL).

The AgNPs were synthesized by mixing a 90 mL solution of 1mM silver salts (acetate/nitrate) with 10 mL of beech/spruce bark extracts. The pH was adjusted (at pH value 9) with NaOH. Afterward, the solutions were maintained at 60°C, in an ultrasonic bath, for the synthesis of the AgNPs. The biosynthesis was considered complete when the color modification appeared, and it was confirmed by UV_VIS analyses. Sterile filters (0.45 μm pore diameter) were used to sterilize the final solutions. Following this protocol, we obtained four AgNPs solutions: silver nanoparticles obtained with beech bark extract and silver acetate (AgNP BBE Ac), 2.16 mg/mL; silver nanoparticles obtained with beech bark extract and silver nitrate (AgNP BBE Nit), 2.33 mg/mL; silver nanoparticles obtained with spruce bark extract and silver acetate (AgNP SBE Ac), 2.93 mg/mL, and silver nanoparticles obtained with spruce bark extract and silver nitrate (AgNP SBE Nit) 2.56 mg/mL.

Gentamicin synergy test - checkerboard method

The checkerboard method was performed for each bacterial strain and for each tested AgNPs, to evaluate if the tested substances exert synergistic activity in combination with different concentrations of gentamicin. Two folded dilutions were performed from the tested AgNPs solutions with Mueller Hinton Broth 2X, in a finale volume of 100 μL, in each horizontal row of a 96-well microtiter plate. From gentamicin sulfate 600 IU/mg (Sigma-Aldrich, United States) we performed dilutions to obtain a gentamicin stock solution of 32, 16, 8, 4, 1, 2, 0.5, 0,25 μg/mL and 50 μL of each dilution were added to each well of each horizontal row of the microtiter plate. Twenty microliters of a 0.5 McFarland bacterial inoculum were mixed with 9980 mL of Muller Hinton Broth 2X (Oxoid, United Kingdom), and from this suspension, 50 μL were added to each well of the 96-microtiter plate. After adding the 50 μL gentamicin solution and the 50 μL of bacterial inoculum, the final volume of each well was 200 μL in all the wells of the microtiter plate, so in the first well of each row, the final concentration of the tested solutions was 50%, while in the last well of each row the final concentration was almost zero (0.0005%). In all the wells of the first row, the final concentration of gentamicin was 8 μg/mL, while in all the wells of the last row the final concentration was almost zero (0.06 μg/mL). Minimum inhibitory concentration for each substance was considered on the well where no visible growth was observed, at the lowest concentration of the other substance. The growth control well was considered the well where both the tested substances and the gentamicin were at their lowest concentrations, while the negative control was considered the well where both the tested substances and the gentamicin were at their highest concentrations. The following formula was used for the assessment of the fractional inhibitory concentration (FIC): FIC = FIC A + FIC B. FIC A was the gentamicin MIC from the assessed well/gentamicin MIC, and FIC B was the tested solution MIC from the assessed well/tested solution MIC. FIC values of 0.5 and below were interpreted as synergistic activity, FIC values between 0.51 – 2 were considered as an indifferent effect, while FIC values higher than 2, were interpreted as antagonistic activity.

The influence of AgNPs on the bacterial growth rate

The MIC wells of each tested substance (registered at the checkerboard method) were reproduced in 10 mL volume, in Muller Hinton Broth, and incubated at 37°C, for 24 hours. For the bacterial growth control, tubes without the

tested substances were used. The optical density of the bacterial suspensions was assessed at 0, 6, 9, 12, and 24 hours, at 600 nm wavelength, using a spectrophotometer (Eppendorf BioPhotometer D30, Eppendorf Austria).

Results

Gentamicin synergy test - checkerboard method

After performing the synergy test, synergistic effects were observed for *E. coli*, for combination of gentamicin 0.25mg/L with AgNP BBE Nit (0.145mg/mL) and with AgNP SBE Ac (0,09mg/mL). The other combinations of different concentrations of tested substances presented indifferent effects with different concentrations of gentamicin.

Overall, the AgNP BBEs solutions in the combination with gentamicin presented lowest values of FIC (0.5, 0.56, 0.75) than the FICs registered for the combination of AgNP SBEs with gentamicin (0.5, 0.63, 1, 1.12), for both bacterial strains.

For *S. aureus* (Figure 1), the combination of gentamicin with both AgNP BBEs solutions presented lowest value of FIC (0.75) than the combination of gentamicin with both AgNP SBEs. The 0.75 FIC value was registered for the following combination: 0.135 mg/mL AgNP BBE Ac with 0.125 mg/L gentamicin, 0.073 mg/mL AgNP BBE Nit with 0.25 mg/L gentamicin and 0.145 mg mg/mL AgNP BBE Nit with 0.125 mg/L gentamicin.

For *E. coli* (Figure 2), the lowest FIC value (at the indifferent effect) was 0.56 was for the combination of 0.036 mg/mL AgNP BBE Nit with 0.5 mg/L gentamicin, followed by a FIC value of 0.57 for the combination of 0.038 mg/mL AgNP BBE Ac with 0.5 mg/L gentamicin and a FIC value of 0.63 for the combination of 0.045 mg/mL AgNP SBE Ac with 0.5 mg/L gentamicin.

The influence of the AgNPs on the bacterial growth rate

The growth rate of the *S. aureus* (Figure 3) was inhibited by all the tested AgNPs at the measurement time points (6, 9, 12, 24 hours), compared to the growth control. The growth inhibition of the *S. aureus* was more pronounced in the presence of AgNP BBE Ac, Ag NP BBE Nit, and AgNP SBE Ac (with almost similar growth rates for these three solutions) than the growth rate inhibition observed in the presence of the AgNP SBE Nit.

After 6 hours, the growth rate of *E. coli* (Figure 4) was inhibited in different degrees by all the tested AgNPs, especially by the AgNP BBE Nit, where the growth rate was almost stationary. At 9 hours, the growth rate of *E. coli* was inhibited only by the Ag NP BBE Ac, AgNP SBE Ac, and AgNP BBE Nit, while the growth rate in the presence of Ag NP SBE Nit was stimulated. Still, the trend of the growth rate of *E. coli* in the presence of AgNP BBE Nit was slower than in the presence of the rest of the AgNPs. After 12 hours of incubation, the growth rates in the presence of the AgNP SBE Ac and AgNP BBE Nit were still

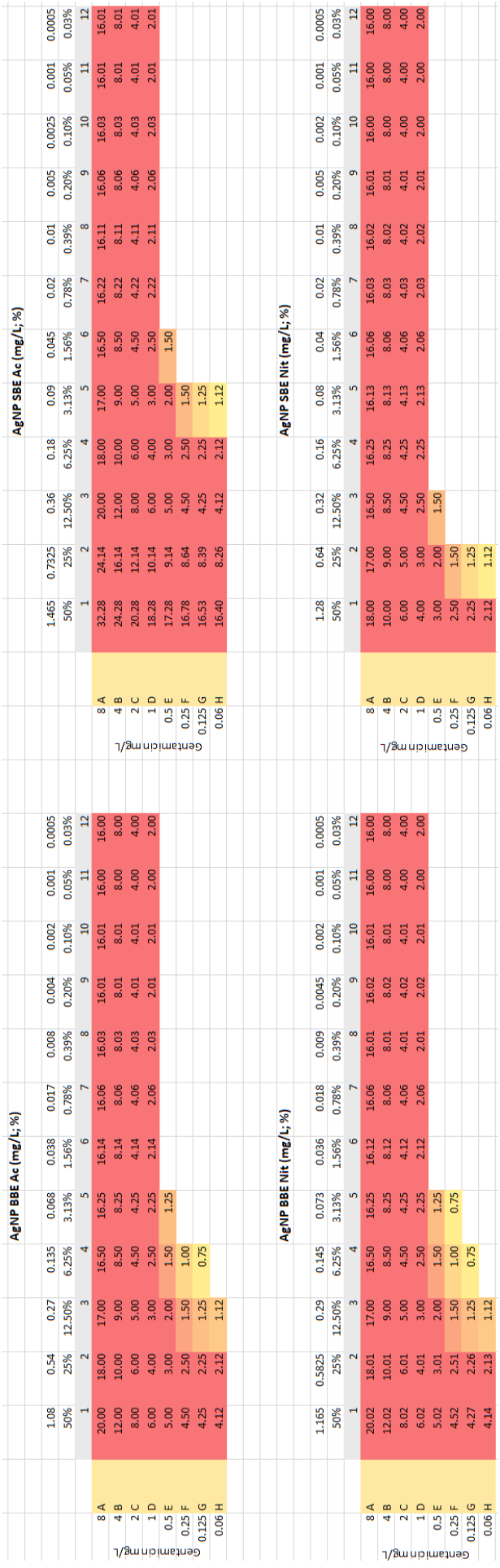

Fig. 1. The synergy tests result for *S. aureus*, FIC values for different combinations of the tested substances with different gentamicin concentrations. The cells highlighted with green represent synergistic activity (FIC values of 0.5 and below); the cells highlighted with yellow represent indifferent effect (FIC values between 0.51 – 2); the cells highlighted with red represent antagonistic activity (FIC values higher than 2).

AgNP BBE Ac (mg/L; %)

Gentamicin mg/L		1.08	0.54	0.27	0.135	0.068	0.038	0.017	0.008	0.004	0.002	0.001	0.0005
		50%	25%	12.50%	6.25%	3.13%	1.56%	0.78%	0.39%	0.20%	0.10%	0.05%	0.03%
		1	2	3	4	5	6	7	8	9	10	11	12
8	A	10.00	9.00	8.50	8.25	8.13	8.07	8.03	8.01	8.01	8.00	8.00	8.00
4	B	6.00	5.00	4.50	4.25	4.13	4.07	4.03	4.01	4.01	4.00	4.00	4.00
2	C	4.00	3.00	2.50	2.25	2.13	2.07	2.03	2.01	2.01	2.00	2.00	2.00
1	D	3.00	2.00	1.50	1.25	1.13	1.07						
0.5	E	2.50	1.50	1.00	0.75	0.63	0.57						
0.25	F	2.25	1.25	0.75									
0.125	G	2.13	1.13										
0.06	H	2.06	1.06										

AgNP SBE Ac (mg/L; %)

Gentamicin mg/L		1.465	0.7325	0.36	0.18	0.09	0.045	0.02	0.01	0.005	0.0025	0.001	0.0005
		50%	25%	12.50%	6.25%	3.13%	1.56%	0.78%	0.39%	0.20%	0.10%	0.05%	0.03%
		1	2	3	4	5	6	7	8	9	10	11	12
8	A	12.07	10.03	9.00	8.50	8.25	8.13	8.06	8.03	8.01	8.01	8.00	8.00
4	B	8.07	6.03	5.00	4.50	4.25	4.13	4.06	4.03	4.01	4.01	4.00	4.00
2	C	6.07	4.03	3.00	2.50	2.25	2.13	2.06	2.03	2.01	2.01	2.00	2.00
1	D	5.07	3.03	2.00	1.50	1.25	1.13	1.06	1.03	1.01	1.01	1.00	
0.5	E	4.57	2.53	1.50	1.00	0.75	0.63						
0.25	F	4.32	2.28	1.25	0.75	0.50							
0.125	G	4.19	2.16	1.13									
0.06	H	4.13	2.09	1.06									

AgNP BBE Nit (mg/L; %)

Gentamicin mg/L		1.165	0.5825	0.29	0.145	0.073	0.036	0.018	0.009	0.0045	0.002	0.001	0.0005
		50%	25%	12.50%	6.25%	3.13%	1.56%	0.78%	0.39%	0.20%	0.10%	0.05%	0.03%
		1	2	3	4	5	6	7	8	9	10	11	12
8	A	10.00	9.00	8.50	8.25	8.13	8.06	8.03	8.02	8.01	8.00	8.00	8.00
4	B	6.00	5.00	4.50	4.25	4.13	4.06	4.03	4.02	4.01	4.00	4.00	4.00
2	C	4.00	3.00	2.50	2.25	2.13	2.06	2.03	2.02	2.01	2.00	2.00	2.00
1	D	3.00	2.00	1.50	1.25	1.13	1.06	1.03	1.02	1.01	1.00		
0.5	E	2.50	1.50	1.00	0.75	0.63	0.56						
0.25	F	2.25	1.25	0.75	0.50								
0.125	G	2.13	1.13	0.62									
0.06	H	2.06	1.06										

AgNP SBE Nit (mg/L; %)

Gentamicin mg/L		1.28	0.64	0.32	0.16	0.08	0.04	0.02	0.01	0.005	0.002	0.001	0.0005
		50%	25%	12.50%	6.25%	3.13%	1.56%	0.78%	0.39%	0.20%	0.10%	0.05%	0.03%
		1	2	3	4	5	6	7	8	9	10	11	12
8	A	10.00	9.00	8.50	8.25	8.13	8.06	8.03	8.02	8.01	8.00	8.00	8.00
4	B	6.00	5.00	4.50	4.25	4.13	4.06	4.03	4.02	4.01	4.00	4.00	4.00
2	C	4.00	3.00	2.50	2.25	2.13	2.06	2.03	2.02	2.01	2.00	2.00	2.00
1	D	3.00	2.00	1.50	1.25	1.13	1.06	1.03	1.02	1.01			
0.5	E	2.50	1.50	1.00									
0.25	F	2.25	1.25										
0.125	G	2.13	1.13										
0.06	H	2.06	1.06										

Fig. 2. The synergy tests result for *E. coli*, FIC values for different combinations of the tested substances with different gentamicin concentrations. The cells highlighted with green represent synergistic activity (FIC values of 0.5 and below); the cells highlighted with yellow represent indifferent effect (FIC values between 0.51 – 2); the cells highlighted with red represent antagonistic activity (FIC values higher than 2).

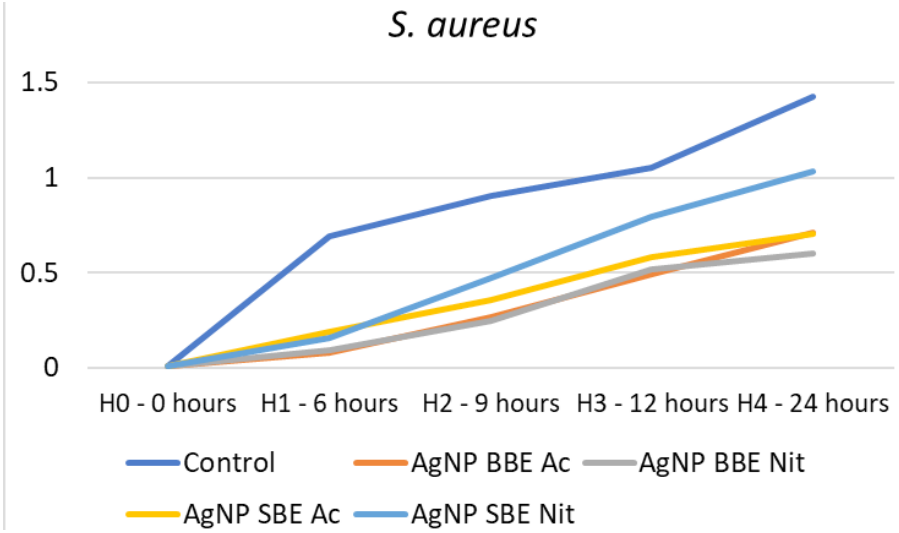

Fig. 3. The growth rate of S. aureus in the presence of the MICs of the tested AgNPs

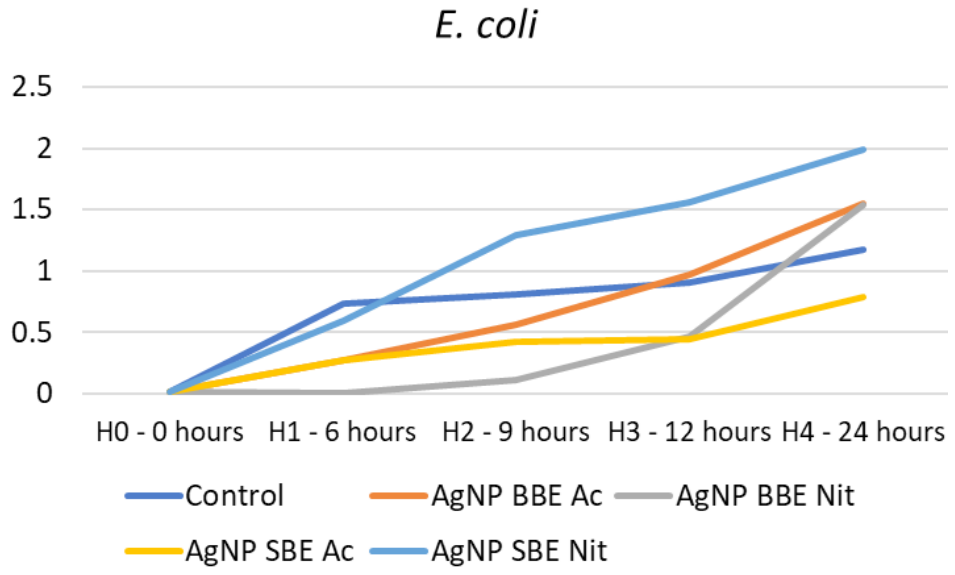

Fig. 4. The growth rate of E. coli in the presence of MICs of the tested AgNPs

inhibited, in the presence of AgNP BBE Ac it was similar to the control growth rate, while the in the presence of the AgNP SBE Nit the growth rate was stimulated. After 24 hours of incubation, only the growth rate in the presence of AgNP SBE Ac was inhibited.

Discussions

Among the multitude of metallic nanoparticles, silver nanoparticles are considered the most effective ones. Biosynthesized AgNPs were reported by several studies as a promising therapeutic alternative, because of their antibacterial, antifungal, and antiviral activity. The green synthesis of nanoparticles using different plant extracts is considered an excellent biosynthesis strategy because of the plant's proprieties (nontoxic, natural capping substances, antimicrobial activity) [18,26]. Also, because the Ag NPs may present multiple associated antibacterial mechanisms, it is unlikely for bacteria to develop resistance against multiple mechanisms at the same time, so the bacterial resistance against AgNPs is considered a rare phenomenon [27–29]. Even so, several recent studies investigated if the bacteria may develop resistance/tolerance against different AgNPs. These studies suggest that bacteria may become tolerant or may develop resistance to sublethal or even lethal doses of different AgNPs, that only phenotypic bacterial changes may be needed to reduce their antibacterial activity, or that antibiotic efficacity may be decreased by the microorganism's prior exposure to the AgNPs [29–33]. An alternative to the possible development of bacterial resistance to AgNPs is considered the additive effect of plant extracts from the biosynthesized nanoparticles, or even the possibility of Ag NPs to exert synergistic activity with different antibiotics [29].

A recent study [34] demonstrated significant synergistic effects for the combination of Ag NPs with different antibiotics, for different bacterial strains. They presented that S. *aureus* was resistant to AgNPs (synthesized using silver nitrate and D-glucose), erythromycin, clindamycin, tetracycline, ampicillin, cefpodoxime, cefuroxime, but when combined with 1 μg/mL of AgNPs the effectiveness of these antibiotics increased from resistance to the susceptible range. The same study presented an increased zone of inhibition of *S. aureus* for the AgNPs combination with gentamicin, while for *E. coli* the inhibition zone was slightly decreased. Another study [35], reported that gentamicin significantly increased the antibacterial effect of poly(N-vinyl-2-pyrrolidone) coated silver nanoparticles against *S. aureus* and *E. coli*. In our study, it was observed that, for *E. coli*, synergistic effects were registered for the combination of gentamicin 0.25mg/L with AgNP BBE Nit (0.145mg/mL) and with AgNP SBE Ac (0,09 mg/mL). All the other combinations of different concentrations of tested substances presented indifferent effects with different concentrations of gentamicin. Overall, the AgNP BBEs solutions in the combination with gentamicin presented lowest values of FIC than the FICs registered for the combination of AgNP SBEs with gentamicin, for *S. aureus* and *E. coli*.

As the antibacterial activity of the Ag NPs BBE and Ag NPs SBE was previously demonstrated [21,25], we considered that it could be an interesting approach to evaluate how these AgNPs might influence the bacterial growth rate over 24 hours. The growth rate of the *S. aureus* was inhibited by all the tested AgNPs at all the measurement times (6, 9, 12, 24 hours), compared to the growth control. The growth inhibition of the *S. aureus* was almost similar in the presence of AgNP BBE Ac, Ag NP BBE Nit, and AgNP SBE Ac, more pronounced than the growth rate inhibition observed in the presence of the AgNP SBE Nit. In the case of *E. coli*, after 24 hours of incubation, the growth rate was inhibited only in the presence of AgNP SBE Ac. An interesting observation was that after 6 hours of incubation, the growth rate of *E. coli* was inhibited in different degrees by all the tested AgNPs, especially by the AgNP BBE Nit, where the growth rate was almost stationary. The well-recognized adaptability of *E. coli* under different environmental conditions might be a possible explanation for this phenomenon. After a slower growth rate of *E. coli* in the presence of AgNPs in the first hours, it was able to adapt to the presence of Ag NPs, and the growth rate was no longer notably reduced, even enhanced in the case of AgNP SBE Nit.

Because of the novelty of the domain and the significant differences between the studies (different methodologies, different types, diffrent concentration of AgNPs, different bacterial strains, etc.) until now, the recently available data only revealed a small part of the multitude of antimicrobial mechanisms of the AgNPs and raised some interesting hypothesis that needs more comprehensive studies to fully elucidate how AgNPs acts against bacteria.

Conclusions

After performing the synergy test, it was observed that, for *E. coli*, synergistic effects were registered for the combination of gentamicin 0.25mg/L with AgNP BBE Nit (0.145mg/mL) and with AgNP SBE Ac (0,09mg/mL). Overall, the AgNP BBEs solutions in the combination with gentamicin presented lowest values of FIC than the FICs registered for the combination of AgNP SBEs with gentamicin, for both bacterial strains.

The growth rate of the *S. aureus* was inhibited by all the tested AgNPs, while for *E. coli*, after 24 hours of incubation, only the growth rate in the presence of AgNP SBE Ac was inhibited. After 6 hours of incubation, the growth rate of *E. coli* was almost stationary in the presence of MIC of AgNP BBE Nit.

The results from this study, as well as the enormous variety of data from the literature, emphasize once again that the green synthesis of AgNPs is a valuable choice for the synthesis of substances that might be used as adjuvants or even as treatment options in the constant battle with bacterial adaptability. More studies are necessary, still, to

completely elucidate the antibacterial mechanisms of these AgNPs and how they might be used for medical purposes.

Authors' contriburion

ADM: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing/review and editing, supervision, project administration, funding acquisition
AM: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing/review and editing
FT: Conceptualization, methodology, resources, writing/review and editing, supervision
BT: Conceptualization, methodology, resources, writing/review and editing
LB: Conceptualization, methodology, resources, writing/review and editing
CT: Conceptualization, methodology, resources, writing/review and editing, supervision
CNC: Conceptualization, methodology, validation, formal analysis, investigation, resources, writing/review and editing, supervision, project administration

Conflict of interest

None to declare.

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