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ANTIBACTERIAL ACTION OF CHEMICAL AND GREEN SILVER NANOPARTICLES IN COMBINATION WITH ANTIBIOTICS

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In the work the synergistic antibacterial activity of chemical silver nanoparticles (AgNPs) in combination with tetracycline and ampicillin, as well as green AgNPs with benzylpenicillin were studied. The tests were performed against the wild strain *E. coli* DSM 1116. Both chemical as well as green AgNPs exhibited antibacterial effect depended on concentration. Our results suggest that chemical AgNPs form complexes with ampicillin and tetracycline. Dose-dependent inhibition of *E. coli* growth is observed for AgNPs only and in complexes with tetracycline. The synergistic antibacterial effect is likely due to enhanced bacterial binding by AgNPs, which is assisted by tetracycline. The inhibitory effect of green AgNPs with benzylpenicillin on the growth of *E. coli* exhibits at concentration of 0.5 mg/ml, has a synergistic effect, which reduces the dose of the antibiotic by 8 times. This concentration of silver nanoparticles is not cytotoxic.

Chemical and green silver nanoparticles – ampicillin – benzylpenicillin – tetracycline – E. coli DSM 1116

Աշխատանքում ուսումնասիրվել է քիմիական նանոմասնիկների հակամանրէային ազդեցությունը ամպիցիլինի և տետրացիկլինի հետ համատեղ, ինչպես նաև կանաչ նանոմասնիկների ազդեցությունը բենզիլպենիցիլինի հետ: Ուսումնասիրություններն իրականացվել են *E. coli* DSM 1116 վայրի շտամի վրա: Արծաթի ինչպես քիմիական այնպես էլ կանաչ նանոմասնիկները ցուցաբերում են կոնցենտրացիայից կախված հակամանրէային ակտիվություն: Արդյունքները ցույց են տալիս, որ արծաթի նանոմասնիկները հակաբիոտիկների հետ առաջացնում են համալիրներ: *E. coli*-ի աճի արգելակումն արծաթի նանոմասնիկների և տետրացիկլինի համալիրի հետ կրում է չափաբաժնից կախյալ բնույթ: Արծաթի նանոմասնիկների և տետրացիկլինի համակցված ազդեցությունը կրում է սիներգիստիկ բնույթ: Կանաչ նանոմասնիկների արգելակիչ ազդեցությունը բենզիլպենիցիլինի հետ նույնպես սիներգիստիկ է, սկսած 0.5 մգ/մլ-ից, ինչն ութ անգամ նվազեցնում է հակաբիոտիկի չափաբաժինը: Նանոմասնիկների այս կոնցենտրացիաները ցիտոտոքսիկ չեն:

Արծաթի քիմիական և կանաչ նանոմասնիկներ – ամպիցիլին – տետրացիկլին – բենզիլպենիցիլին – E. coli DSM 1116

В работе изучалась совместная антибактериальная активность химических наночастиц серебра (AgNP) с ампициллином и тетрациклином, а также зеленых AgNP с бензилпенициллином. Исследования проводились на диком штамме *E. coli* DSM 1116. Как химические, так и зеленые AgNPs проявляли антибактериальный эффект в зависимости от концентрации. Результаты показывают, что химические AgNPs образуют комплексы с ампициллином и тетрациклином. Дозозависимое ингибирование роста *E. coli* наблюдается

только для AgNP и в комплексах с тетрациклином. Синергетический антибактериальный эффект проявляется при комбинированном действии AgNP с тетрациклином. Ингибирующее действие зеленых AgNPs с бензилпенициллином на рост *E. coli* проявляется начиная с концентрации 0.5 мг/мл, имеет синергетическое действие, которое снижает дозу антибиотика в 8 раз. Эта концентрация наночастиц серебра не цитотоксична.

Химические и зеленые наночастицы серебра – ампициллин – тетрациклин – бензилпенициллин – E. coli DSM 1116

Infectious diseases are one of the leading causes of death worldwide. World health organization (WHO) expressed serious concern about the continued development of multidrug resistance of bacteria, among which *E. coli* has extreme priority [9]. The lack of new antimicrobials is associated with an increase in antibiotic resistance [7, 10, 13]. This has triggered worldwide initiatives to develop new and more effective antimicrobial compounds, as well as new delivery and targeting strategies [14, 21], including nanostructured materials. Nanoparticles, such as silver nanoparticles (AgNPs) [2, 5, 17, 19] can be used to deliver antimicrobial compounds and they can also serve as antipathogenic agents, as well as inhibit biofilm formation and stop population threshold (quorum sensing), thus suppress the expression of pathogenicity genes and multidrug resistance. Other strategies include the combined use of herbal antimicrobials and nanoparticles [4, 16] to overcome toxicity problems. NPs synthesized using plant extracts (green NPs) are less toxic in comparison to other methods. The use of green NPs can reduce the toxicity and side effects associated with high systemic concentrations of drugs [8, 12, 20]. β-lactam antibiotics of different generations are intensively studied for their biological and organic aspects. As for the bio-inorganic chemistry of these antibiotics, that is, their interaction with metals and the ability to form complexes have not been adequately studied. It has been shown that the combination of amoxicillin with AgNPs has greater bactericidal activity against *Esherichia coli* than when they are used separately [11, 15, 18]. Synergistic effects between AgNPs and polymyxin B have been shown for Gram-negative *E. coli* [3, 22].

It is known that tetracycline inhibits the binding of aminoacyl-tRNA to the A site of the ribosome and can alter the cytoplasmic membrane of bacteria, causing leakage of intracellular contents, such as nucleotides from the cell [8].

In this study we examined the antibacterial mechanism of two different classes of conventional antibiotics: β-lactam benzylpenicillin (BP), ampicillin (Amp) and polyketide tetracycline (Tet) in combination with chemical and green AgNPs, against the wild strain of Gram negative bacteria *Escherichia coli* DSM 1116.

Materials and methods. As a colloid solution of AgNPs we used "Biocidal Additive" produced by Concern "Nano-industry" (Moscow, Russia). Chemical AgNPs had a spherical shape with a diameter of 4 to 24 nm, stabilized by 3-(trimethylsilyl)-1-propanesulfonic acid sodium salt (DSS). *E. coli* DSM 1116 wild-type bacterium (Russian-Armenian university collection) has been used for the experiments, which is a natural lysogenic strain stored at a temperature of 3-5°C. Laboratory stock of *E. coli* strain was grown in peptone (2 % peptone, 0.5 % NaCl) medium buffered with 0.1 M K_2HPO_4 (pH 7.5), and 0.2 % glucose was added. For the preparation of the solid medium a 1.5 % agar was added to the liquid medium [17]. The bacterial growth rate was determined by measuring the changes in optical density (OD) of bacterial suspension at a wavelength of 625 nm and monitored every hour till 8 h and at 24 h.

Plant extract of *Ocimum araratum* was added to the silver nitrate solution. The transition of Ag^+ to Ag^0 was confirmed by the alteration of the color of the solution from colorless to brown. SEM (SEMLEO-1430 VP, Carl Zeiss, Germany) was used for the detection of the forms and types of NPs. The UV–Vis absorption spectra were recorded using a spectrophotometer from 200 nm to700 nm after mixing for 0, 2 and 24 h relative to deionized water. The results indicate that AgNPs were formed during the "green" synthesis.

Susceptibility of bacteria to NPs and antibiotics were observed on the basis of standardized agar diffusion zones (halos), which can indicate bacterial growth inhibition [6]. Bacterial suspension of 100 µl was disseminated on plates; disks with deposited NPs were placed on agar and incubated in 37 °C for 24 h. After the incubation agar halos were observed and diameters were measured using "Image Repair 3" programme [17].

To allow full exposure of bacteria to the antibiotic or the antibiotic in combination with AgNPs the sample mixtures were gently shaken in the incubator shaker at 25 ºC for either 30 min or 2 h. Thereafter, aliquots were transferred and evenly spread onto tryptic agar plates at about 1×10^8 cells/plate. All plates were incubated for 24 h at 37 ºC. Colony Forming Units (CFUs) were counted with a plate counter. AgNPs and antibiotics were tested separately as controls. Each concentration was tested with four plates and the average values were recorded as CFU/plate.

Each experiment was repeated 8 to 12 times. The graphs and diagrams show the arithmetic mean and their standard errors ($p<0.05$).

Results and Discussion. Chemical AgNPs have a characteristic absorption at around 395 nm, while Amp has no absorption in the range of 300–700 nm (fig. 1). The addition of Amp to AgNPs solution causes a reduction in AgNPs' extinction at 395 nm. This demonstrates that Amp forms a complex with AgNPs, which leads to aggregation of AgNPs. Complex formation was observed with a precipitation and color change when the two solutions were mixed. On the absorption spectra, the AgNPs peak is completely lost. All optical density measurements were made with respect to deionized water and DSS. Note that all spectra also were measured after incubation for 24 hours under dark conditions.

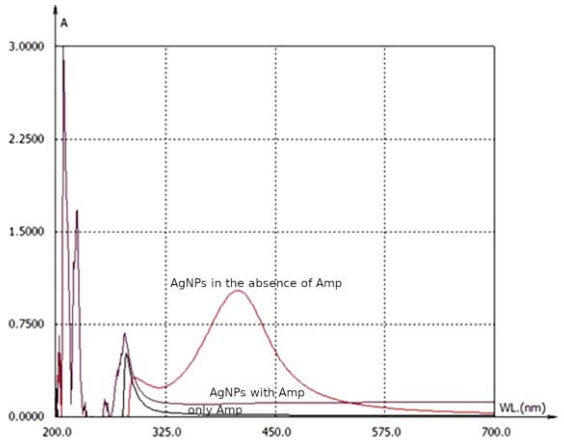

Fig. 1. UV–Vis spectra of chemical AgNPs in the absence and presence of Amp, only Amp.

Tet has an absorption extending to 500 nm and interferes with the absorption peak of AgNPs at high concentrations.

To obtain green silver NP, a 50 % ethanol extract of *Ocimum araratum* was chosen due to the fact that the preliminary screening of antiradical activity (ARA) and total content of flavonoids (TCF) of different extracts revealed the maximum value for this particular extract. Further, the physical characteristics of the obtained NPs were investigated using SEM (data not shown) and spectral analysis (fig. 2).

The effect of chemical AgNPs on the growth of the *E. coli* bacterium was investigated by disc-diffusion. Both AgNPs and Amp inhibit the growth of *E. coli* DSM 1116. When combined with a β-lactam antibiotic – Amp, the growth inhibition at the

highest concentration of Amp is less than 35 % (fig. 3). AgNPs at a concentration of 0.105 μg/ml exhibited weak antibacterial effect, and a strong antibacterial effect at a concentration of 0.21 μg/ml.

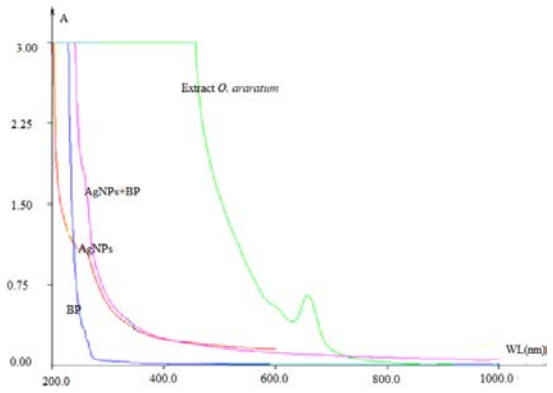

Fig. 2. UV–Vis spectra of tested samples (*O. araratum* extract; AgNPs; BP; AgNPs+BP).

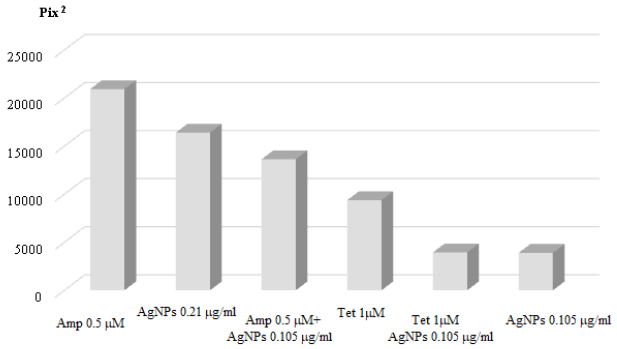

Fig. 3. Areas of inhibition zones under the influence of AgNPs and antibiotics on *E. coli*, $p<0.05$.

Therefore, there is no synergistic effect of Amp when combined with AgNPs against *E. coli* DSM 1116, the effect of Amp decreased. AgNPs in combination with Tet reduced its antibacterial properties (fig. 3). The investigation of green AgNPs action on the growth of the *E. coli* bacterium by disc-diffusion method revealed that AgNPs at concentrations of 0.25 mg/ml, 0.5 mg/ml and 1 mg/ml and dispersed in peptone, suppressed the growth with lysis zones 99071; 103847; 154519 pixel^2 (fig.4). Extract of *O. araratum* did not possess antibacterial properties. The effect of green AgNPs and NPs stabilized in the extract on the growth of *E. coli* confirmed that the lysis zones were mediated only by the action of AgNPs.

AgNPs and AgNPs stabilized in the extract, as well as antibiotics (separately and in combination with AgNPs) on the formation of *E. coli* CFU were also investigated.

AgNPs without extract and stabilized in the extract at high concentrations (0.5 mg/ml –1 mg/ml) completely suppressed the growth of CFU of *E. coli*. But at a concentration of 0.25 mg/ml, the activity of AgNPs against the growth of *E. coli* microcolonies was 7.5 times higher compared to the effect of stabilized NPs.

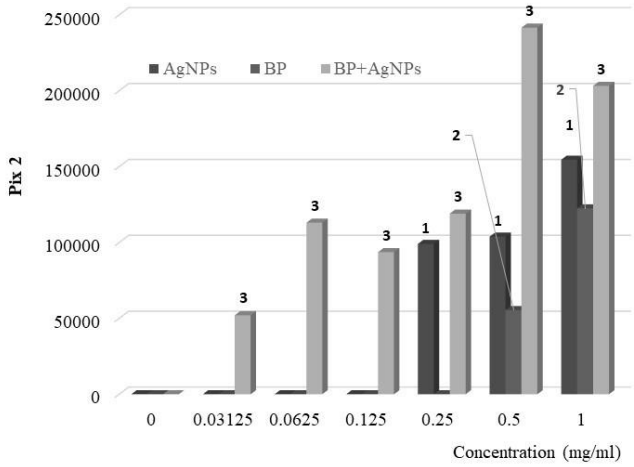

Fig. 4. Areas of lysis zones of *E. coli* under influence of AgNPs (1) and BP (2) and AgNPs combined with BP (3), $p<0.05$.

Exposure to chemical AgNPs alone at 0.21 μg/ml and 0.105 μg/ml causes 35 % and 24 % inhibition of CFU (fig. 5). However, the combination of Tet with 0.105 μg/ml AgNPs causes 22 % decrease of CFU in the bacteria. Tet itself does not have an inhibitory effect on CFU. The number of colonies increased when AgNPs (0.105 μg/ml) were combined with Amp (0.5 μg/ml).

Fig. 5. CFU of *E. coli* after exposure to Tet or Amp combined with AgNPs: control, AgNPs, Amp, Amp + AgNps, Tet + AgNps (from left to right).

Benzylpenicillin, regardless of the concentration, does not inhibit the growth of the formation of *E. coli* CFU. The effect of AgNPs in combination with BP at high concentrations completely inhibited the growth of colonies. BP, at a concentration of 0.25 mg/ml, in combination with AgNPs suppressed the growth of microcolonies (fig. 6). AgNPs in combination with BP showed high activity against bacterial growth, while BP did not.

Growth of *E. coli* bacteria in suspension under the influence of antibiotics and AgNPs are described in [17]. Amp did not inhibit the growth of *E. coli*. Tet ~~slows~~ slowed down the bacterial growth (5 and 10 μM) and completely inhibited the growth of *E. coli* at concentrations of 20 and 50 μM. The inhibition by Tet is concentration dependent. AgNPs either did not suppress growth, and with increasing concentration (50 to 100 μM), the growth was suppressed.

The growth kinetics of *E. coli* was carried out with the addition of AgNPs and antibiotics at various concentrations.

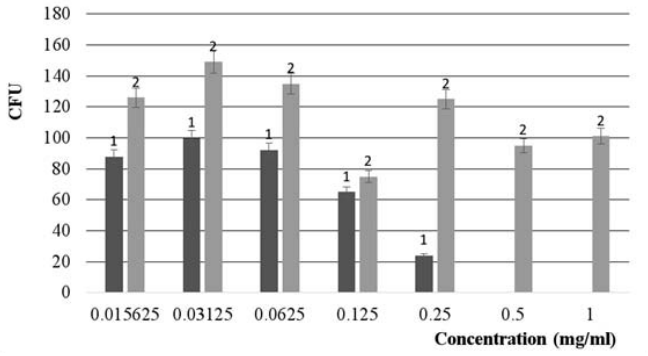

Fig. 6. The action of AgNPs without (1) and with BP (2) CFUs of *E. coli*, $p<0.05$.

According to literature data, the duration of the log phase determines the antibacterial activity of the substance [1]. The log phase of the control sample lasted 2 hours, for BP – 3 hours, AgNPs – 4 hours. Consequently, the most active is AgNPs, then BP (fig. 7).

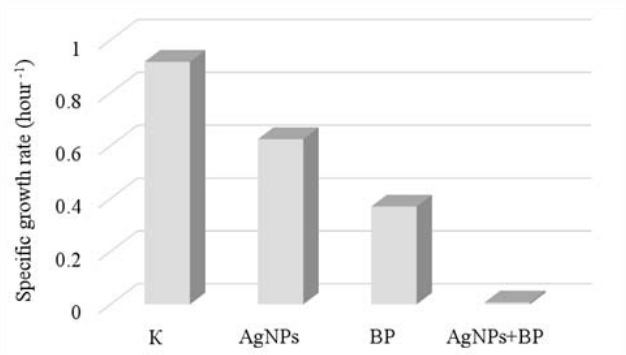

Fig. 7. Combined action of green AgNPs and BP on the kinetic of growth of bacteria, $p<0.05$.

Thus the antibacterial properties chemical AgNPs in combination with Amp are reduced. In addition to precipitation, the characteristic peak of the NPs disappears completely. The instability of the Amp molecule leads to a very rapid decrease in its effectiveness against bacteria. In the case of AgNPs in combination with Tet the antibacterial effect was reduced by disk-diffusion method, but the effect of the latter is enhanced by the growth of CFU. We hypothesize that the combined antibacterial activity correlates with the complex formation between AgNPs and antibiotics. Amino group in Amp allows the penetration into the outer membrane of Gram-negative bacteria. Our results showed that AgNPs are the most active in combination with the tested antibiotics and can exhibit both an indifferent and a synergistic dose-dependent effect with a decrease in the MIC of antibiotics. It is important to note that synergistic effects of AgNPs in combination with non tropic antibiotics (Tet, Amp and BP) against *E. coli* bacteria are observed. The AgNPs concentrations are very low and not cytotoxic for human erythrocytes.

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