SILVER AND GOLD NANOPARTICLES AND QUATERNARY AMMONIUM SALTS FOR THE DISINFECTION OF SARS-CoV-2

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In the context of the current Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic, a very common problem is the choice of optimal disinfectant for the decontamination / disinfection of contaminated surfaces, medical salons, medical equipment, multi-purpose protective equipment, medical instruments and areas frequented by persons.

The paper presents tests for possibly contaminated the virucidal and bactericidal effect of new solutions based on silver and gold nanoparticles (AgNPs / AuNPs) and quaternary ammonium salts against SARS-CoV-2, by a surrogate method that quantifies the cytopathic effect of a Sabin attenuated LSc/2ab strain of poliovirus on an immortal rhabdomyosarcoma cell line.

Keywords: SARS-CoV-2, AgNPs, AuNPs, disinfection, surrogate method

1. Introduction

Coronavirus disease 2019 (COVID-19) caused by Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) is an ongoing global health emergency. Since 31 December 2019 and as of 01 December 2020, over 63 million cases of COVID-19 (in accordance with the applied case definitions

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and testing strategies) have been reported, including 1.4 million deaths [1, 2]. Our most important line of defense is our own immune system, however people with at least one underlying co-morbidity are highly vulnerable and their only line of defense is sanitizers, face masks, immune system boosters and drugs that are clinically approved. Scientists around the world have made promising strides towards developing approaches to prevent COVID-19 [3, 4].

Chemical disinfectants effective against a wide variety of pathogens have been used for disinfection of personal protective equipment and surfaces. Despite promising results from chemical disinfectants, they are often associated with disadvantages such as high concentration requirements for 100% viral inhibition or limited effectiveness over time [5].

Nanotechnology could play a very important role on the current pandemic when implemented in developing highly effective nano-based antimicrobial and antiviral formulations that are not only suitable for disinfecting air and surfaces, but are also effective in reinforcing personal protective equipment such as facial respirators [7, 6]. Nanomaterials present an enormous potential as disinfectants against coronaviruses, mainly due to unique attributes of nanomaterials including intrinsic anti-viral properties.

This paper presents virucidal and bactericidal efficiency tests of chemical disinfectants based on quaternary amonium salts [8] and gold and silver nanoparticles solutions [9]. The virucidal tests were performed by a surogate method that quantifies the cytopathic effect of a Sabin attenuated LSc/2ab strain of poliovirus on an immortal rhabdomyosarcoma cell line, results that can be quantified to coronaviruses.

2. Experimental work

2.1. Samples preparation

For this experimental work 7 solutions were prepared for the virucidal and bactericidal tests, consist of 4 nanoparticles solutions and 3 organic solutions.

The organic solutions prepared are based on 4th generation of quaternary ammonium salts, didecyl dimethyl ammonium bromide (DDDMABr) in concentrations of 0.5%, 1% and 1.5% in water.

For the nanoparticles solutions, 4th types of samples with a noble metal content were synthesized, as follow: 3 types of silver nanoparticles (AgNPs) with and without functionalization, of different sizes and shapes (quasi-spherical and nanoplates) and 1 type of functionalized spherical gold nanoparticles (AuNPs).

The syntheses were performed by methods that cumulatively meet the following requirements: a) efficient control of the size and shape of the obtained nanoparticles; b) minimum reagent consumption/low cost; and c) procedure with minimal impact on human organisms and for environmental. The AgNPs were

synthesized by electrochemical method of non-functionalized AgNPs, electrochemical method stabilized with xanthan gum AgNPs, chemical synthesis of anisotropic AgNPs and the AuNPs were synthesized by classical chemical method stabilized with xanthan gum. 4 solutions were proposed for characterization: S1–spherical AuNPs 0.25 mg/L; S2–spherical AgNPs 0.50 mg/L; S3-spherical AgNPs 0.25 mg/L; S4-nanoplates AgNPs 0.01 mg/L;

In order to evaluate the performance of solutions containing NPs, it is necessary to identify and apply efficient testing methods. The properties, behavior and biological effects of nanomaterials (NMs) can be influenced by a number of physicochemical parameters. Detailed NMs characterization information and data can also provide a quick assessment of the risk they may pose. The information on the obtaining process can lead to the physico-chemical and morphological evaluation of the NM, can also provide information on impurities. The behavior, interaction and effects of NMs are inevitably influenced by nanodimensions (size, morphology, surface), the nature of the chemical substance, including surface characteristics and structural form. At the same time, NMs may pose health and/or environmental risks not only due to their chemical composition, but also due to their nano-size, surface composition, which may modulate absorption, biokinetics and toxic effects. In this regard, it is important to note that any nano-related properties are intrinsically related to the physical integrity of the nano-structure.

For the NPs samples characterization, techniques for determining the surface plasmon resonance (SPR), the zeta electrokinetic potential of nanoparticles and scanning electron microscopy (SEM) were applied.

Investigations to determine surface plasmon resonance were performed using the Orion AquaMate 8000 UV-VIS Spectrometer, in the wavelength range 200-800 nm, absorbance mode, 0.5 nm scan interval. Plasmon resonance, in the case of spherical AuNPs, ranges from 518 nm to 533 nm with an increase in diameter from 10 to 48 nm. In the case of AgNPs, the absorption peak characteristic of surface plasmon resonance is found at approximately 490 nm and is dependent on the density and size of AgNPs.

Samples S1 - S4 were characterized in terms of particle diameter and zeta potential by laser diffusion technique (DLS), using ZetaSizer NanoZS equipment at 25°C, at a dispersion angle of 90º using a 1 mM NaCl solution.

Measurements of size, intensity and polydispersity index were performed in triplicate. From the data presented in Fig. 1 it is found that samples S1 and S2 contain NPs characterized by bimodal distribution, S3 contains a trimodal distribution, while S4 a monomodal distribution. It is observed that 85.7% particle distribution for S1 is in the 644 nm band while below 14.5% distribution is in the 91.28 nm band. S2 contains particles in the range of 260.8 nm (70% distribution) and 42.6 nm (30% distribution). The NPs volume for S3 shows three average diameters of 491.7 nm (49.8%), 145.1 nm (48.4%) and 6,104 nm (1.8%). Sample

S4 contains a nanoparticle volume of 100% with a size of 107.5 nm and the data presented reveal the lowest polydispersity (0.18%). All samples containing noble metal nanoparticles are stable.

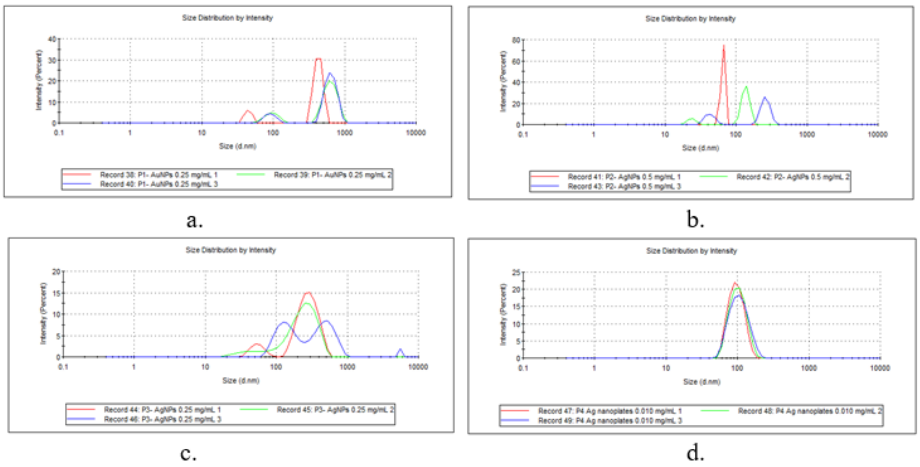


Fig. 1 Particle size distribution as a function of intensity a. S1, b. S2, c. S3, d. S4

SEM analysis was used to identify the morphology and particle size of the analyzed samples. The samples were evaporated at 200°C to separate the solid nanoparticles. SEM micrographs indicates for S1 average dimensions of 30 nm, at a magnitude of 120,000 x, for S2 average dimensions of 40 nm with a spherical shape (magnitude of 200000 x), for S3 dimensions between 94 and 150 nm with a spherical shape (magnitude 100,000 x), and for S4 dimensions of 50 nm (magnitude 100,000 x).

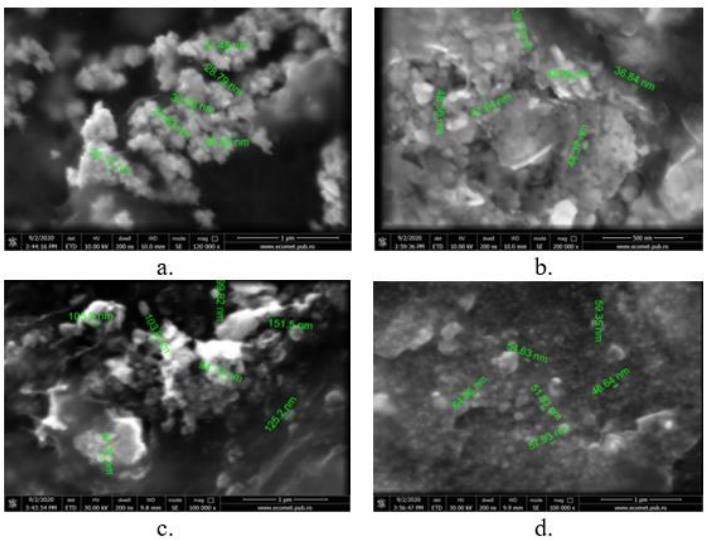


Fig. 2 SEM analysis of samples a. S1, b. S2, c. S3, d. S4

The results are correlated with previous investigations, the larger size being given by the tendency of agglomeration of samples. The elemental composition indicated by EDS analyzes revealed the presence of Au in S1 and Ag in S2, S3 and S4.

2.2. Virucidal and bactericidal efficiency tests

For the virucidal and bactericidal activity, 7 solutions were prepared:
S 1 – AuNPs - Gold spherical nanoparticles solution, 25 nm, 0.25 mg/L;
S 2 – AgNPs - Silver spherical nanoparticles solution, 40 nm, 0.50 mg/L;
S 3 – AgNPs - Silver spherical nanoparticles solution, 90-100 nm, 0.25 mg/L;
S 4 – Nanoplates AgNPs – Silver nanoplates nanoparticles solution, 50 nm, 0.01 mg/L;
S 5 – DDMNBr - Didecyl dimethyl ammonium bromide aqueous solution, 0.5 %
S 6 – DDMNBr - Didecyl dimethyl ammonium bromide aqueous solution, 1 %
S 7 – DDMNBr - Didecyl dimethyl ammonium bromide aqueous solution, 1.5 %

2.2.1. Virucidal efficiency tests

By measuring the cytopathic effect of the virus, one can assess the efficiency of the tested solutions and quantify their virucidal effect. Therefore, indirectly, the efficiency of the solutions on coronaviruses can be appreciated.

Testing of the virucidal effect of the solutions was performed by a surrogate method that quantifies the cytopathic effect of a polio virus strain on an immortal rhabdomyosarcoma cell line.

It was assumed to test the samples for simulated cleaning conditions. For this purpose, a rhabdomyosarcoma (RD) cell line, poliovirus strain 1, LSc 2ab, bovine albumin and test solutions were used.

If the sample examined from the product under test, by the validated method, results in a logarithmic reduction of at least 4 for the solutions used under simulated cleaning conditions - 0.3 g/L bovine albumin, under the required test conditions, for the strain Poliovirus type 1, LSc 2ab is considered that, for general uses, the virucidal concentration is the active concentration - according to SR EN 14476: 2013 + A2: 2019 partially. For this, the rhabdomyosarcoma cell monolayer was prepared one day before titration. The virus titration mix consists of 0.1 mL albumin, 0.1 mL viral suspension (Poliovirus strain type 1, Sabin 1) and 0.8 mL disinfectant solution. The contact time of the mixture was 60 sec. at 20°C. 0.5 mL of the mixture was transferred to 4.5 mL of synthetic cell culture media (MEM 2% SF stored at 4°C). Decimal dilutions of this mix from 10^{-1} to 10^{-8}, cell control (Cc) and virus control (Cv) were prepared. 400 µL diluted mix was inoculated into each well. The plates were read daily, for several days.

Evolution of the cytopathic effect of RD cell infection (derived from rhabdomyosarcoma) by different dilutions of poliovirus (10^{-2}, 10^{-4}, 10^{-5}, 10^{-6}) after

being put in contact with the solution S7 is presented in Fig. 3. Starting with dilution 10^{-6} the effect of the poliovirus was neutralized by the tested solution.

The results of the virucidal effect were extrapolated to coronavirus.

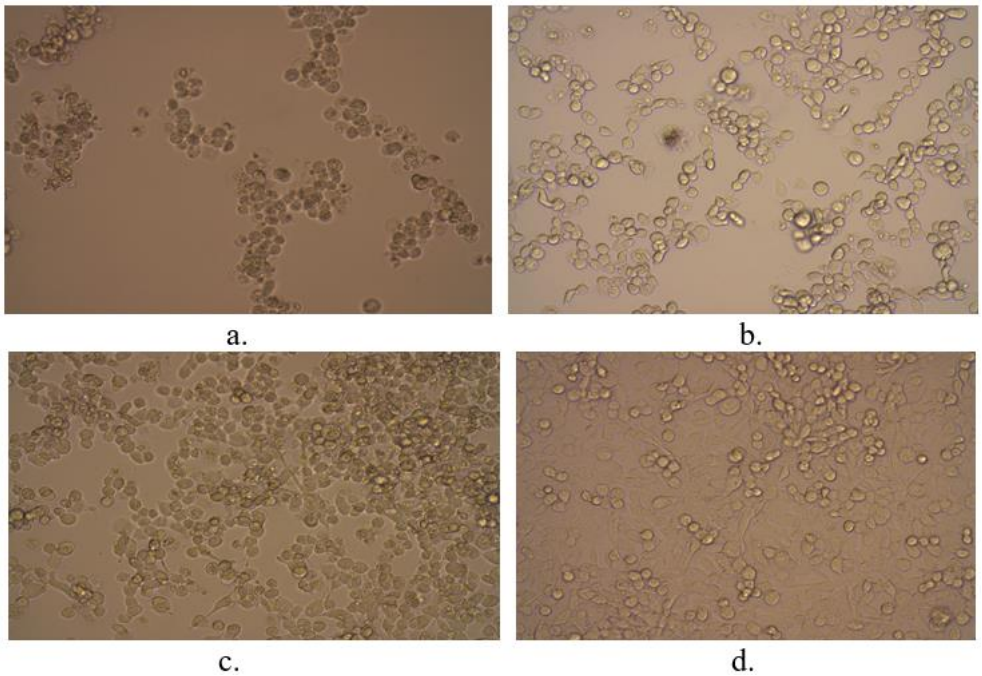


Fig. 3 Microscopic analysis of the mix at dilutions a. 10^{-2}, b. 10^{-4}, c. 10^{-5}, d. 10^{-6}

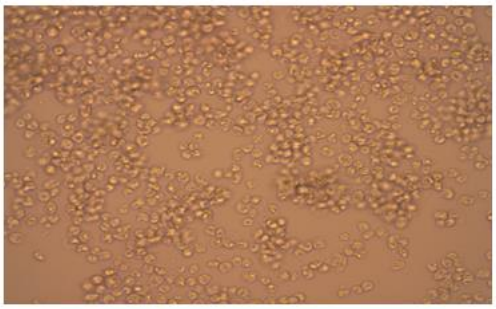


Fig. 4 Aspect of cellular cytotoxicity

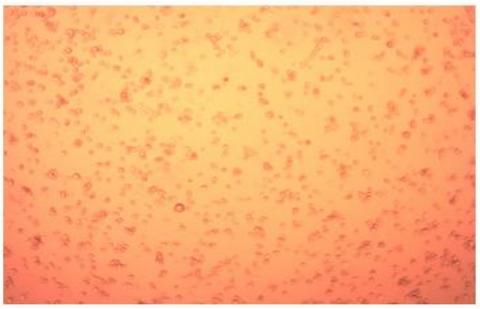


Fig. 5 ECP cytopathic effect - RD cell line

Viral suspension titer used was $TCID_{50}$ log: 7.5. The logarithmic reduction of the virus titer after testing the samples for S5 tested solution was between 3.5-4 log, and for all the other solutions (1,2,3,4,6,7) was ≥ 4 log after the first day of reading the plates. The readings are made up to 5 days.

It can be concluded that the disinfectant solutions have a virucidal effect using the surrogate method, so it can be appreciated that they have a virucidal effect and absorb the SARS-CoV-2 virus.

Except S5, where the virucidal activity was at the lower limit, for all solutions (1,2,3,4,6,7) the presence of virucidal activity is found according to the results presented above.

In order to comply with the standard SR EN - 14476 + A2 *Quantitative testing of the suspension for the evaluation of virucidal activity in the medical field*, additional validation tests are required: Control of the interference of the test product, inactivation tests of the virus with formaldehyde solution as well as the repetition of these determinations but also the testing with other viral strains (adenovirus, etc.) mentioned in the standard. The results demonstrate the feasibility of the idea and the efficiency of the solutions.

2.2.2. Bactericidal efficiency tests

Bacterial strains used are representative species for the main groups of pathogenic bacteria (most common in infectious pathology, hospital flora, nosocomial infections and multidrug-resistant infections): *Staphylococcus aureus* ATCC 25923, for gram-positive shells; *Escherichia coli*, ATCC 25922, for gram-negative bacilli, enterobacteria; *Pseudomonas aeruginosa* ATCC 27853, gram-negative bacilli, nonenterobacteria.

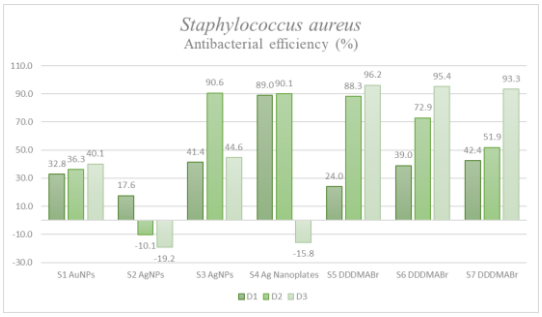
Evaluation *in vitro* of the antimicrobial activity of the nanoparticles and organic solutions were performed by two methods: a quantitative method - the method of microdilution in plates and a qualitative method - difuzimetric antibiogram method (modified Kirby-Bauer technique).

- ***The method of microdilution in plates working protocol***:

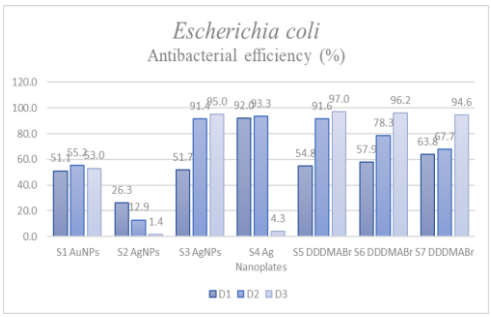
The lyophilized bacterial strains were transferred to the nutrient broth for "rejuvenation" for 24 hours at 37°C, then cultured on specific agar media by transplanting into "open polygon" and incubated for 24 hours at 37°C. A bacterial suspension in saline with a concentration of 0.5 McFarland turbidimetric units (1.5x108 cells/mL) was prepared from the typical colony, which was used to seed 96-well microplates. In parallel, the strains were checked by microscopy on a Gram stained smear. To compare the antimicrobial effect, the solutions were analyzed in dilutions by testing on the same 96-well microplate, inoculated separately with a bacterial species, each row being reserved for a solution (S1-S7), and the columns as follow: no. 1 blank - saline 200 µL, no. 2 negative controls - saline 50 µL, undiluted solution 50 µL and culture medium 100 µL, no. 3 positive controls - saline 50 µL, bacterial suspension (*Staphylococcus aureus, Escherichia coli, Pseudomonas aeruginosa)* 50 µL and culture medium 100 µL, no. 4, 5 and 6 undiluted solution 50 µL, bacterial suspension 50 µL and culture medium 100 µL, no. 7, 8 and 9 solution at first dilution (dilution 1/10 solution 50 µL, bacterial suspension 50 µL and culture medium 100 µL), no. 10, 11 and 12 solution at the second dilution (dilution 1/100 solution 50 µL, bacterial suspension 50 µL and

culture medium 100 µL). Thus, the effective concentration of the test substance for each solution is decreasing and the results obtained were used to estimate the antimicrobial efficacy. Inoculated and incubated microplates were measured spectrophotometrically with EnSight Multimode Plate Reader computer equipment. The spectrophotometric measurement of the absorbance of the bacterial microcultures put in contact with the test solutions, indicated the bacterial development which was read at 562 nm and 600 nm (primary data). The most sensitive result was at 600 nm, where the highest general arithmetic mean of the absorption was obtained.

Negative values indicate favoring bacterial growth. If the bacteria has multiplied in the culture medium, it means that the solution has no antibacterial effect and a high absorbency is recorded. This compares with the reference Absorbance (Positive Control Abs + Negative Control Average Abs - Blank Absorbance) expressed as a percentage and represents the effect of the substance on bacterial growth. The inverse of this percentage shows the antibacterial efficiency of the respective solution, at different concentrations. Based on these calculations was determined the antibacterial efficiency of the tested solutions. The interpretation of antibacterial efficacy at mathematical intervals was quantified as follows:



a.



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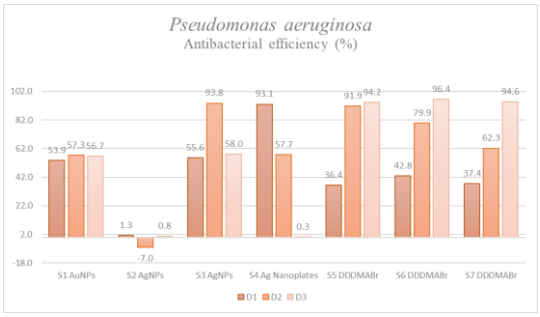

c.

Fig. 6 Antibacterial efficiency of the tested solutions with a. *Staphylococcus aureus*, b. *Escherichia coli*, c. *Pseudomonas aeruginosa*

- ***Difuzimetric antibiogram method working protocol:***

The lyophilized bacterial strains were passed on nutrient broth for "rejuvenation", for 24 hours, at 37°C, then they were cultured on specific agar media, by transplanting in "open polygon" and incubated for 24 hours, at 37°C. A bacterial suspension in saline with a concentration of 0.5 McFarland turbidimetric units was prepared from the typical colony, which was used to seed the Muller Hinton agar plates "in canvas" using a sterile buffer. In the test were introduced: Negative control (un impregnated sterile paper disc) – M; Samples: sterile paper disc impregnated with 10 µL test solution - 1; sterile paper disc impregnated with 20 µL test solution - 2; borosilicate glass cylinder with 100 µL test solution - 3.

Testing the antimicrobial activity of the 7 solutions after reading at 24 h revealed the appearance of the halo specific to the difuzimetric activity in most of the tested solutions: S3, S4, S5, S6 and S7.

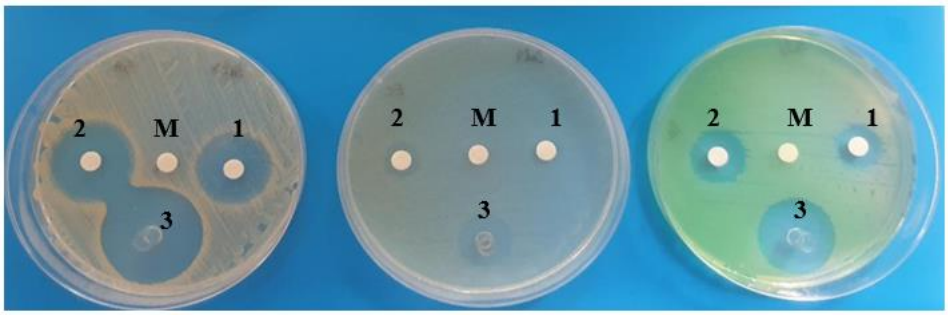

Fig. 7 Testing the antimicrobial activity of S4 by contact with *Staphylococcus aureus* (left), *Escherichia coli* (center), *Pseudomonas aeruginosa* (right) - 24 h reading to determine the presence of the inhibition zone around the samples

S1 and S2 did not show antimicrobial activity. Solutions 3, 4, 5, 6 and 7 showed antimicrobial activity, the strongest effect being highlighted in solution 4, on all 3 bacterial species used in the test.

3. Conclusions

The current Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2) pandemic challenged the scientific world to find new ways to fight against spreading it, by decontamination / disinfection and protection.

The present paper summarizes the bactericidal and virucidal efficiency tests of 4 solutions of gold and silver nanoparticles (AuNPs / AgNPs) with and without functionalization, with different sizes and shapes, and 3 aqueous solutions based on 4th generation of quaternary ammonium salts, of didecyl dimethyl ammonium bromide in different concentrations.

The virucidal effect of the solutions against SARS-CoV-2 was performed by a surrogate method that quantifies the cytopathic effect of a Sabin attenuated LSc/2ab strain of poliovirus on an immortal rhabdomyosarcoma cell line. In order to comply with the standard SR EN - 14476 + A2 Quantitative testing of the suspension for the evaluation of virucidal activity in the medical field, additional validation tests are required. The results demonstrate the feasibility of the idea and the efficiency of the solutions.

The bactericidal effect was demonstrated by a quantitative method - the method of microdilution in plates and a qualitative method - difuzimetric antibiogram method, in 5 of 7 solutions.

Acknowledgements

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R E F E R E N C E S

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