



Antimicrobial Activity of Ag/Ag₂O Nanoparticles Synthesized by Dead Biomass of Yeast and their Biocompatibility with Mammalian Cell Lines

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Abstract: The high frequency of resistance of pathogenic bacteria and fungi to commercial antimicrobial agents has become an alarming public health threat. We report a versatile option for antimicrobial therapy, through recycling metal pollutants, using Ag/Ag₂O nanoparticles (NPs) naturally synthesized by dead biomass of the yeast *Rhodotorula mucilaginosa* (*R. mucilaginosa*), a nano-biosorbent isolated from the wastewater of a mine in the Amazon region. In *in vitro* assays, the Ag/Ag₂O NPs exhibited promising antimicrobial activity against Gram-positive Methicillin-resistant *Staphylococcus aureus* (MRSA) and Gram-negative multidrug-resistant (MDR) *Escherichia coli* (*E. coli*) strains, and against the yeast *Cryptococcus neoformans* (*C. neoformans*), as demonstrated by the disc diffusion method, minimum inhibitory concentration (MIC), minimal bactericide concentration (MBC) and minimum fungicidal concentration (MFC). The atomic force microscopy (AFM) revealed complete destruction of the microorganisms by the Ag/Ag₂O NPs. The toxicity assays of the Ag/Ag₂O NPs in mammalian cells demonstrated reduced cytotoxicity, an important characteristic in biomedical application. The Ag/Ag₂O NPs synthesized by the nanobiosorbent *R. mucilaginosa* exerts a potent inhibitory effect on microorganisms and is safe for eukaryotic cells, providing a possible application to nanomedicine and, at the same time, recycling polluting material.

Keywords: Silver nanoparticles, dead biomass, bacteria, yeast, antimicrobial activity, mammalian cell lines, wastewater, mine.

1. INTRODUCTION

Outbreaks of infectious diseases caused by different pathogenic bacteria and fungi and the development of antimicrobial resistance have led to the search for new antimicrobial agents [1]. A literature review demonstrated remarkable bactericidal, fungicidal and antiviral activity of silver NPs [2]. The chemical and physical methods currently used for the synthesis of NPs are expensive, require high temperatures and high pressures, and produce hazardous wastes that possess risk to the environment [3]. In this respect, biological methods for the nanomaterials synthesis are used worldwide to create nano-factories [4], particularly for the preparation of silver NPs that offer several benefits such as compatibility for pharmaceutical and biomedical application [5]. Yeasts have become important in nanotechnology as producers of NPs, due to their advantages such as low-cost, rapid growth and easy preparation and biomass handling. Salvadori *et al.* [6] described for the first time the efficient and low-cost synthesis of Ag/Ag₂O NPs using dead biomass of the yeast *R. mucilaginosa*, isolated from the wastewater of a mine in the Amazon region, for nanobioremediation of silver waste and its reuse in NPs form.

The MRSA are important pathogenic Gram-positive bacteria in the healthcare setting that continue to persist in the hospital and community. In humans, *S. aureus* causes central nervous system infections, invasive endocarditis, urinary tract infections, superficial skin lesions and localized abscesses, osteomyelitis, septicemia, septic arthritis, and pneumonia [7]. More than half of all infections in vascular surgery are caused by MRSA [8].

Resistance to three or more antimicrobial agents in the absence of intrinsic resistance is defined as multidrug resistance. The MDR among clinical isolates of bacteria such as *E. coli*, is an important healthcare problem and is associated with a global increase in morbidity and mortality [9].

The encapsulated saprophytic yeast, *C. neoformans* var *grubii* (capsular serotype D) causes 82% of cryptococcal diseases in the world [10]. Var. *neoformans* (capsular serotype A) accounts for 20%–30% of HIV-associated cryptococcal meningitis in northern Europe [11]. Decaying organic matter, soil and avian guano are the main environmental reservoirs of both subtypes of *C. neoformans* [10].

The antimicrobial potential of silver NPs is due to the strong oxidative activity of their surface and the release of silver ions into biological environments. An important advantage of biogenic NPs is that they are less cytotoxic than commercial silver NPs or silver ions [12].

In a previous study, we reported for the first time the use of dead biomass of the yeast *R. mucilaginosa* isolated from a mine in Amazon region for nanobioremediation and intracellular synthesis of metallic copper NPs [13]. The same system was used for the nanobioremediation and production of magnetic Ni/NiO NPs in the form of nanostructured films [14] and, recently, with metal silver [6]. In the present study, we explore for the first time effectiveness of Ag/Ag₂O NPs, synthesized by the nanobioremediator (dead biomass of yeast *R. mucilaginosa*) [6], as an antibacterial and antifungal against representative microorganisms of public health concern. The safety of these NPs was demonstrated by their low cytotoxicity in mammalian cells, increasing the choice of low-cost, safe and effective antimicrobials.

2. MATERIALS AND METHODS

2.1. Biosynthesis of Biogenic Ag/Ag₂O Nps

R. mucilaginosa was isolated from water collected in a copper waste pond of the Sossego mine, which belongs to the Company Vale S.A. located in Canaã dos Carajás, Pará, Brazilian Amazonia region (0°26'S latitude and 50°4'W longitude) [13]. Biosynthesis of the Ag/Ag₂O NPs by the yeast was performed according to Salvadori *et al* [6].

2.2. Bacterial Strains

The Gram-positive *Staphylococcus aureus* MRSA ST5 and Gram-negative *Escherichia coli* BL-II-11 (MDR) strains, were obtained from the Bacterial Culture Collection of the Bacteriology Section of the Department of Microbiology, Institute of Biomedical (ICB), University of São Paulo. For all experiments, the strains were cultured in Mueller-Hinton (MH) medium.

2.2.1. Determination of the Antibacterial Activity of Ag/Ag₂O Nps By the Disc Diffusion Method

The antibacterial activity of the Ag/Ag₂O NPs was evaluated by the diffusion disc method commonly used for antibiotics. The bacterial strains were inoculated (10^8 CFU mL⁻¹) into plates with MH agar (Oxoid, England) and paper discs (5 mm in diameter) instilled with different concentrations (1, 2, 5, 10, 20, 50, 70 and 100 µg mL⁻¹) of Ag/Ag₂O NPs were laid on the inoculated test microorganism. The plates were incubated at temperature of 37°C during 24 h. Antimicrobial activity was determined by measuring the inhibition zone around the disc.

2.2.2. Determination of the Antibacterial Effectiveness of Ag/Ag₂O NPs by the MIC Assay

The antibacterial activity of the Ag/Ag₂O NPs was evaluated based on the minimum inhibitory concentration (MIC), using the standard broth tube dilution method [15]. The MIC was determined against a bacterial concentration of 10⁶ CFU mL⁻¹ cultured in MH broth (Oxoid, England) with different concentrations of Ag/Ag₂O NPs (1, 2, 5, 10, 20, 50, 70 and 100 µg mL⁻¹). The cultures were incubated at 37°C, during 24 h at 250 rpm. The bacterial concentrations were determined by measuring optical density (OD) at 600 nm. The MIC was defined as the lowest concentration of NPs that inhibited bacterial growth.

2.2.3. Determination of the Antibacterial Activity of Ag/Ag₂O NPs by the MBC Assay

The minimum bactericidal concentration (MBC) of the Ag/Ag₂O NPs was determined using the same solutions as in the tube dilution test of the MIC assay [16], which were inoculated into MH agar plates at 37°C for 24 h. The bactericidal effect of different Ag/Ag₂O NPs concentrations was evaluated by the absence of colonies on the agar plate. The MBC was defined as the lowest concentration of Ag/Ag₂O NPs that totally inhibited bacterial growth when compared to the positive control (no treatment with Ag/Ag₂O NPs).

2.3. Yeast Strain

The yeast *Cryptococcus neoformans* H-99, was obtained from the Fungal Culture Collection of the Micology Section of the Department of Microbiology, ICB, University of São Paulo. For all experiments, the strain was cultured in Sabouraud Dextrose (SD) medium.

2.3.1. Determination of Antifungal Activity of Ag/Ag₂O NPs by the Disc Diffusion Method

The disc diffusion method was used to evaluate the *in vitro* antifungal activity of Ag/Ag₂O NPs against *C. neoformans*. Petri dishes containing Sabouraud Dextrose Agar (SDA) (Oxoid, England) were inoculated with the fungal strain (10⁶ CFU mL⁻¹) and paper discs (5 mm in diameter) instilled with different concentrations (0.1, 0.15, 0.2, 0.5, 1, 2, 5, 10, 20, and 40 µg mL⁻¹) of Ag/Ag₂O NPs were laid on the inoculated test microorganism. The plates were incubated at temperature of 35°C during 48 h and antimicrobial activity was determined by measuring the inhibition zone around the disc.

2.3.2. Determination of the Antifungal Activity of Ag/Ag₂O NPs by the MIC Assay

The antifungal activity of the Ag/Ag₂O NPs was also evaluated based on the MIC, using the standard broth tube dilution method [15]. The MIC was determined against a fungal concentration of 10⁶ CFU mL⁻¹ cultured in Sabouraud Dextrose Broth (SDB) (Oxoid, England) with different concentrations of Ag/Ag₂O NPs (0.1, 0.15, 0.2, 0.5, 1, 2, 5, 10, 20, and 40 µg mL⁻¹). The cultures were incubated at 35°C, during 48 h at 250 rpm. The fungal concentrations were determined by measuring optical density (OD) at 600 nm. The MIC was defined as the lowest concentration of NPs that inhibited the growth of the yeast.

2.3.3. Determination of the Antifungal Activity of Ag/Ag₂O NPs by the MFC Assay

The minimum fungicidal concentration (MFC) of the Ag/Ag₂O NPs was determined using the same solutions as in the dilution test of the MIC assay [16], which were inoculated into SDA plates at 35°C for 48 h. The fungicidal effect of different Ag/Ag₂O NPs concentrations was evaluated by the absence of colonies on the agar plate. The MFC was defined as the lowest concentration of Ag/Ag₂O NPs that totally inhibited fungal growth when compared to the positive control (no treatment with Ag/Ag₂O NPs).

2.4. Atomic Force Microscopy of Bacterial and Yeast Cells After Treatment with Ag/Ag₂O Nps

Changes in untreated bacterial and yeast cells and cells treated with Ag/Ag₂O NPs were investigated by Atomic-force microscopy (AFM). Samples were scanned with an AFM Icon Nanoscope V (Bruker) using Tapping mode AFM technique with oscillating cantilever to scan the sample surface with scan rate of 1 Hz and a silicon nitride tip with resonance frequency of about 250 kHz.

2.5. Cytotoxicity Assays with Ag/Ag₂O Nps

BeWo cells (human placental choriocarcinoma), A-253 cells (human submaxillary salivary gland), KNS-42 cells (human glioma) and Vero cells (african green monkey kidney) were obtained from the Laboratory of Human and Animals Enteric Viruses, Department of Microbiology, ICB, University of São Paulo. The KNS-42 and Vero cells were cultured at 37°C in Dulbecco's Modified Eagle Medium-high glucose (DMEM) (Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich, St. Louis, MO, USA). A-253 and BeWo cells were cultured in McCoy medium (Sigma-Aldrich, St. Louis, MO, USA) and F-12K medium (Sigma-Aldrich, St. Louis, MO, USA), respectively, under the same conditions as described for the other cells. For the evaluation of cytotoxic activity [17], 100 µL medium and 1.5x10⁵ cells per well were added to the wells of 96-well plastic plates. After 24 h, culture medium was aspirated and 100 µL of fresh medium specific for each cells without FBS and different concentrations of Ag/Ag₂O NPs were added to each well. The plates were incubated at 37°C in a 5% CO₂ atmosphere. Morphological changes were observed over a period of 48 h. Untreated cells were used as positive control. Cell viability was evaluated by the Alamar Blue (AB) assay (Thermo Fisher Scientific, Waltham, MA, USA), which assesses cell viability based on the reduction potential of metabolically active cells. Briefly, following the 48 h incubation, 10 µL of AB solution was added to each well. After 4 h incubation at 37°C, the optical density was then measured at the excitation and emission wavelengths of 570 and 600 nm, respectively, and the results were read using a fluorescence spectrophotometer (GloMax®-Multi Detection System - Promega Corporation).

3. RESULTS AND DISCUSSION

The present study used a synthetic biological system consisting of dead biomass of the yeast *R. mucilaginosa* as a nano factory to synthesize an antimicrobial Ag/Ag₂O NPs. Initially this synthetic biological system (dead biomass of the *R. mucilaginosa*) become a natural tool to performing functions for cleaning environmental as metals nanobiosorbent from wastewater [6,13,14], and synthesise concomitantly in NPs with high aggregated comercial value [6,13,14]. And now as the principal focus of the present study, we use these Ag/Ag₂O NPs with a spherical shape and average size of 11 nm as an antimicrobial against bacteria and fungi and analysed their cytotoxicity in mammalian cells.

3.1. Antimicrobial Activity of Ag/Ag₂O Nps

Silver NPs widely recognized for their antimicrobial activity [18]. In this respect, Ag/Ag₂O NPs synthesized by dead yeast biomass may be a novel choice of agents for antimicrobial therapy, showing promising results in both bacteria and yeast.

E. coli MDR and MRSA were challenged with Ag/Ag₂O NPs concentrations of 1 to 100 µg/ml. Both *E. coli* MDR and MRSA were inhibited by a concentration over 1 µg/ml, in the disc diffusion test (Fig. 1). Table 1 shows the diameter of the inhibition zones at the different concentrations of Ag/Ag₂O NPs. The bacteriostatic effect of Ag/Ag₂O NPs was evaluated based on their MIC, which was the same for both bacterial species (Table 2). The decrease in absorbance observed at a concentration of NPs higher than 1 µg/ml, suggests that cell death occurred in the stationary phase. Analysis of the number of colonies formed on the MH agar plates showed bactericidal effect of the NPs was dose dependent in all assays, and no bacterial growth of either species was observed at NPs concentrations higher than 1µg/ml in the MBC assays. The Ag/Ag₂O NPs exhibited a high antibacterial activity for both bacteria when compared with results already described in the literature for antibiotics commercially available and also metal NPs of the biogenic origin [12,19].

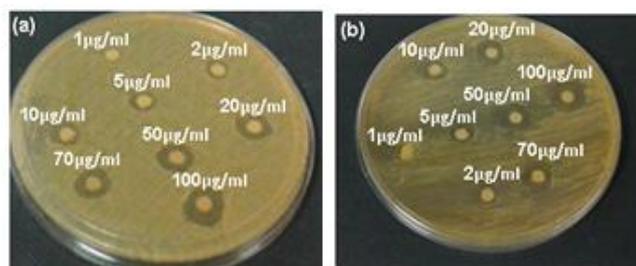


Figure1. Antibacterial activity of Ag/Ag₂O NPs against against *E. coli* MDR (a) and MRSA (b) determined by the disc diffusion method.

Table1. Inhibitory activity from disc diffusion method of Ag/Ag₂O NPs against *E. coli* MDR and MRSA.

Bacteria	Ag/Ag ₂ O NPs Concentrations (µg/ml)	Inhibition zone (mm)
<i>E. coli</i> MDR	1	Resistant
	2	11
	5	12
	10	14
	20	16
	50	17
	70	18
	100	20
MRSA	1	Resistant
	2	11
	5	13
	10	15
	20	17
	50	19
	70	20
	100	22

Table 2. MIC of Ag/Ag₂O NPs against *E. coli* MDR and MRSA.

Bacteria	Ag/Ag ₂ O NPs Concentrations (µg/ml)	Absorbance (OD 600nm)
<i>E. coli</i> MDR	Control (without NPs)	2.3
	1	1.4
	2	—
	5	—
	10	—
	20	—
	50	—
	70	—
MRSA	Control (without NPs)	1.1
	1	0.5
	2	—
	5	—
	10	—
	20	—
	50	—
	70	—

The Figures 2 and 3 illustrate the results of AFM analysis of the effect of Ag/Ag₂O NPs on MRSA and *E. coli* MDR, respectively, demonstrating damages to the cells of both bacterial species compared to untreated cells (control). The results of AFM analysis of bacterial cells treated with biogenic silver NPs are relatively recent. The mechanism underlying the inhibitory effect of silver NPs on microorganisms has not been fully elucidated. Studies suggest a direct interaction of silver NPs with the cell membrane, promoting its disruption [20]. Furthermore, an interaction of silver NPs with thiol (-SH) groups found in proteins has been proposed, as well as the production of reactive oxygen species (ROS) [21] or release of silver ions inhibiting respiratory enzymes, also generating ROS [22]. Another study suggested that thiol groups present in prokaryotic cells form complexes with silver NPs, causing the death of these cells [23].

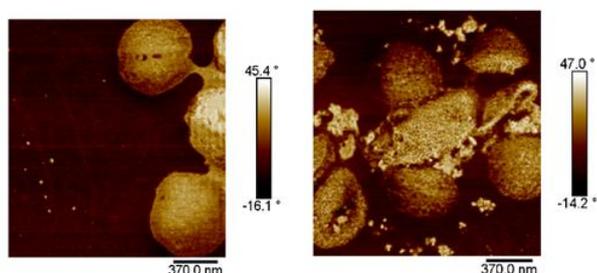


Figure 2. AFM images illustrating the antimicrobial activity of Ag/Ag₂O NPs against MRSA. (a) Image of MRSA control and (b) Image of MRSA cells damaged after treatment with Ag/Ag₂O NPs.

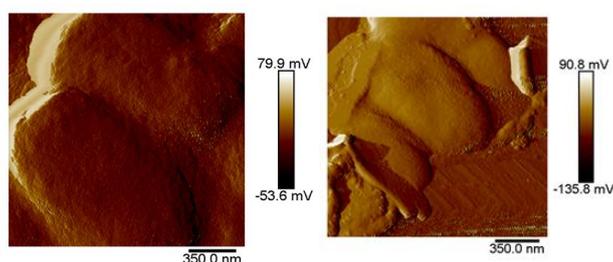


Figure 3. AFM images illustrating the antimicrobial activity of Ag/Ag₂O NPs against *E. coli* MDR. (a) Image of *E. coli* MDR control and (b) Image of *E. coli* MDR cells damaged after treatment with Ag/Ag₂O NPs.

Concentrations of the Ag/Ag₂O NPs of 0.1 to 40 µg/ml were tested by the disc diffusion method and potent antifungal activity against the yeast *C. neoformans* was observed at a concentration higher than 0.15 µg/ml (Fig. 4). Table 3 shows the diameter of the inhibition zones at the different Ag/Ag₂O NPs concentrations, and the results were confirmed by the MIC (Table 4) and MFC. The Ag/Ag₂O NPs exhibited a high efficacy as antifungal against an important pathogen, the yeast *C. neoformans* compared with other biogenic silver NPs [24].

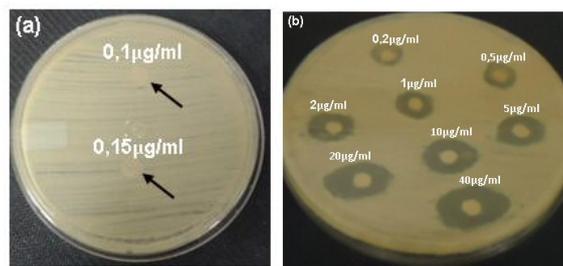


Figure4. Antifungal activity of Ag/Ag₂O NPs against *C. neoformans* determined by the disc diffusion methods. (a) *C. neoformans* resistant to discs impregnated with Ag/Ag₂O NPs at concentrations of 0.1 to 0.15 µg/ml (black arrows). (b) *C. neoformans* susceptible to Ag/Ag₂O NPs at concentrations of 0.2 to 40 µg/ml.

Table3. Inhibitory activity from disc diffusion method of Ag/Ag₂O NPs against *C. neoformans*

Yeast	Ag/Ag ₂ O NPs Concentrations (µg/ml)	Inhibition zone (mm)
<i>C. neoformans</i>	0.1	Resistant
	0.15	Resistant
	0.2	16
	0.5	18
	1	20
	2	22
	5	25
	10	27
	20	29
	40	31

Table4. MIC of Ag/Ag₂O NPs against *C. neoformans*.

Yeast	Ag/Ag ₂ O NPs Concentrations (µg/ml)	Absorbance (OD 600nm)
<i>C. neoformans</i>	Control (without NPs)	1.8
	0.1	1.3
	0.15	1.3
	0.2	—
	0.5	—
	1	—
	2	—
	5	—
	10	—
	20	—
	40	—

The Figure 5 shows the AFM images illustrating the effect of Ag/Ag₂O NPs on the yeast *C. neoformans*. Damaged yeast cells can be observed when compared to untreated cells (control). A recent study proposed that silver NPs exert antifungal effects through apoptosis [25], but the exact mechanism underlying the inhibitory effects of silver ions on yeasts remains to be elucidated.

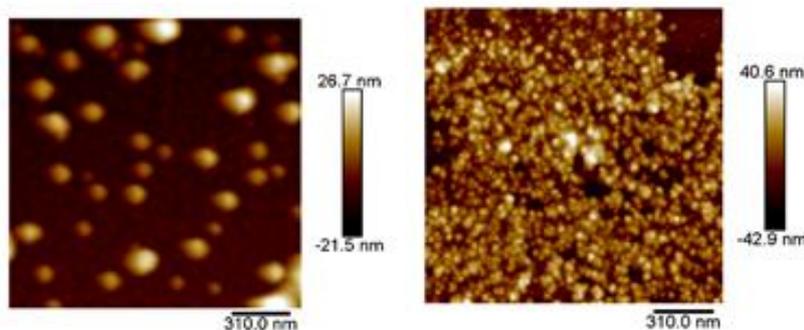


Figure5. AFM images illustrating the antimicrobial activity of Ag/Ag₂O NPs against *C. neoformans*. (a) Image of *C. neoformans* control and (b) Image of *C. neoformans* cells damaged after treatment with Ag/Ag₂O NPs.

3.2. Effect of Ag/Ag₂O Nps on Mammalian Cells

To further elucidate the effect of Ag/Ag₂O NPs on eukaryotic cells, mammalian cells were placed in contact with different concentrations of NPs. The cells were analysed by inverted light microscope and by the AB assay (Fig. 6). Microscope imaging suggests that Ag/Ag₂O NPs do not significantly affect mammalian cells. This was confirmed by the AB assay, especially when the cells were exposed to a low dose yet higher than the dose necessary to achieve antimicrobial activity. The AB assays revealed that BeWo and A-253 cells (human origin) are significantly resistant when treated with high concentrations of Ag/Ag₂O NPs, with an approximate IC₅₀ value of 150 µg/ml (Fig. 6a and b). KNS-42 cells, also of human origin (neuronal cells), and Vero cells were also resistant to Ag/Ag₂O NPs with approximate IC₅₀ values of 70 and 60 µg/ml respectively (Fig. 6c and d). The IC₅₀ values found in our study were similar to or higher than those reported in the literature for silver NPs [12,26].

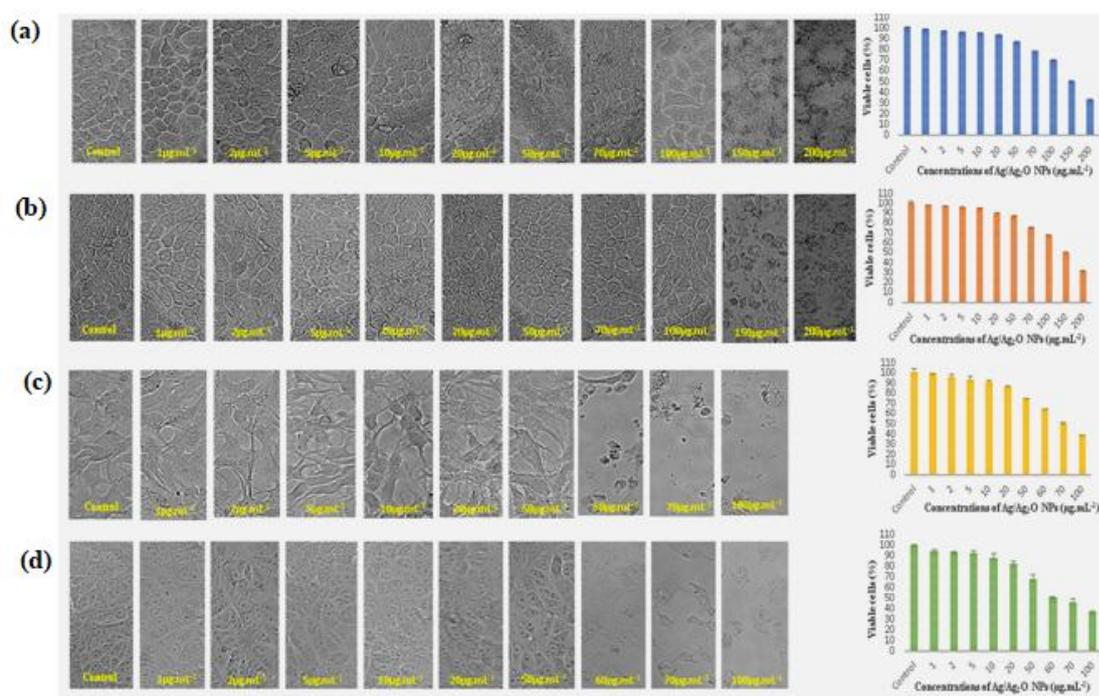


Figure 6. Cytotoxicity assays of different concentrations of Ag/Ag₂O NPs compared to untreated cells (Control), 200X magnification, and evaluated in cells viability tests through AB assays, plotted in graphics beside of the cell panels. (a) BeWo cells; (b) A-253 cells; (c) KNS-42 cells and (d) Vero cells.

4. CONCLUSIONS

This study provides evidence that Ag/Ag₂O NPs synthesized by a natural route (dead yeast biomass) could compete with other biogenic silver NPs and commercial antibiotics indicated for the treatment of infections. Thus, in addition to being derived from the recycling of metal pollutants, assisting in the cleaning of ecosystem, these NPs may also be used as efficient antimicrobial agents to combat infections caused by multidrug-resistant pathogenic microorganisms. However, further studies are needed to elucidate the exact mechanism of action of Ag/Ag₂O NPs on microorganisms, and in vivo experiments in animal models before their use as antimicrobial agents.

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