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Envision of the Microbial Contact with Mycosynthesized Silver Nanoparticles.

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ABSTRACT

The goal of the current work was to visualize the interaction between silver nanoparticles of 8.9-16.73 nm and some microorganisms as well as to estimate the morphological uniqueness of natural-assemblage of some mycosynthesized silver nanoparticles (AgNPs) with different bacteria and fungi, to ascertain the prospect of the antimicrobial potential of the biosynthesized AgNPs that were systematically evaluated against fourteen pathogenic bacteria and eight fungal isolates included four isolates of yeast like fungi. Remarkably, the AgNPs-antimicrobial activity was evident particularly against highly infectious tested isolates of bacteria like methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Alcaligenes faecalis*, *Escherichia coli*, as well as several fungal pathogens of *Candida albicans*, *C. tropicalis, Trichoderma reesei*, *Aspergillus niger,* and *Penicillium marneffei*. Moreover, electron microscopy visualizations of the interactions between AgNPs and tested microorganisms showed that AgNPs were very harmful to some of them causing complete damage to the cells, while it binds compactly to the exterior of some others and/or entered the cell with no observable injures to them, indicating satisfactory natural- assemblage capability. Conclusively, our outcomes showed the way that biosynthesized AgNPs interact with the investigated microorganisms which indicate possibly using the AgNPs as a potent antibacterial and/or antifungal agent against broad spectrum pathogenic bacteria and fungi.

Keywords: Nanobiotechnology, Antimicrobial, MIC, Fungi, Bacteria, AgNPs, TEM, SEM.

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INTRODUCTION

The latest demonstration of nanobiotechnology has granted a new curative character of silver nanoparticles applications employed in medicine. Silver powder was assumed by Hippocrates, father of modern medicine, to possess useful therapeutic and anti-diseases quality that considered as a remedy for ulcers. But it was chiefly silver compounds that really medicinally practiced [2].

Silver compounds were foremost cure for wound diseases in World War I awaiting the discovery of antibiotics. Metallic silver used in new engineering technologies leading to extremely original characteristics. As an alternative of being made "large", metallic silver is transformed into ultra fine particles whose dimension was measured in nanometers (nm) [1].

Principally due to extremely small size, silver particles revealed extraordinarily physicochemical properties and biological activities. These unique properties broaden its appliance in antibacterial, antifungal, anti-viral and anti-inflammatory therapy [3,4].

Microorganisms like bacteria and fungi participate chiefly in bioremediation of toxic metals via reduction of metal ions to nano-forms, so were believed as promising nano-factories. Filamentous fungi are ideal candidates for this purpose. The fungal systems or myco-nanofactories have been demoralized for the fabrication of metal nanoparticles of silver, gold, zirconium, silica, titanium, iron (magnetite) and platinum [9]. In that ledge [10], described the intracellular production of silver nanoparticles (AgNPs) of 2~25 nm within *Verticillium* sp. with the residues of the metal stacked to the outside of the cytoplasmic membrane.

Even if not any of the studies reviewed definitively proved evidence of any instant influence to human wellbeing or the environment by a silver nano-material containing product, the whole science reviews suggested some caution advises and additional investigations were necessary given the actually prevalent and briskly emergent utilize of silver nano-materials [18].

In the present study, we attempted to analyze and visualize the antimicrobial activity of AgNPs synthesized by different fungal isolates against several pathogenic test microorganisms of bacteria, yeasts and fungi. In addition, scanning and transmission electron microscopy were used to assess their efficacy as a potent antibacterial and/or antifungal agent against several pathogenic bacteria and fungi.

EXPERIMENTAL

Preparation of the AgNPs Extract

The tested fungal species; *Verticillium chlamydosporium var. chlamydosporium* CBS600.88, *Aspergillus niger NRRL 595, Trichoderma viride, T. longibranchiatum, Penicillium aurantiogresium* IMI 89372*, P.roqueforti* IMI 285518*,* were previously subjected for the extracellular and intracellular biosynthesis of AgNPs and characterized using UV–Visible Spectral Analysis, FT-IR, EDX, XRD and TEM analyses in an earlier work [19] as illustrated in table 1.

Table 1: Extracellular and Intracellular AgNPs biosynthesized from different fungal species

RCMB Culture-Collection Code	Fungal species	*Type strain Culture-Collection Code*	*Mean size of AgNPs (nm)*	
			Extracellular	*Intracellular*
RCMB039005	*Verticillium chlamydosporium var. chlamydosporium*	CBS600.88	-	8.97
RCMB002007(4)	*Aspergillus niger*	NRRL 595	-	14.48
RCMB017002	*Trichoderma viride*	Soil Isolate	13.47	12.86
RCMB017007	*T. longibranchiatum*	Soil Isolate	11.0	12.95
RCMB001002(1)	*Penicillium aurantiogresium*	IMI 89372	12.70	16.73
RCMB001009(1)	*P. roqueforti*	IMI 285518	15.13	11.70

Microorganisms and Media

About twenty two tested isolates of fourteen bacteria and eight fungal isolates were employed in this research to examine their sensitivity to AgNPs extracts (Table 2). Bacterial isolates, other than type strains, were classified by means of biochemical tests in accordance with the instruction manual of clinical microbiology [20]. While, fungi, other than type strains, were recognized via the macroscopic and microscopic characteristics by means of the culture collection and identification unit of the Regional Center for Mycology and Biotechnology (RCMB), Al-Azhar University, Cairo, Egypt. The examined bacterial and fungal isolates were preserved on nutrient agar (Merck, Germany) and Sabouraud dextrose agar slants (Merck, Germany), respectively, at 4°C in anticipation of investigation.

Antimicrobial Sensitivity Using Agar -Well Diffusion Assay

All AgNPs extracts were prepared in final concentrations of 6.8 mg/ml for antimicrobial tests. The prime screening antimicrobial tests were performed utilizing agar-well diffusion technique [20] using 100 µl of suspension containing 10^8 CFU/ml of bacteria or 10^5 spore/ml of fungi spread evenly on the surface of the nutrient agar and Sabouraud dextrose agar plates, respectively, just after wells were cut evenly at the peripherals and the center of the Petri plates. 10 µl of AgNPs extract solution was dispensed in each well. 10 µl of both mycelia-free water extract and 2mM $AgNO_3$ (Sigma, Germany) were used as negative and positive controls, respectively. The plates were incubated for 24 h at 37°C for bacterial growth, and for 5-7 days at 30-35°C for fungal growth. Clear inhibition zones around wells point to the attendance of antimicrobial activity. For more accurate results, all experiments were done in triplicates.

Determination of Minimum Inhibitory Concentrations (MIC)

For the most AgNPs-sensitive tested isolate, MIC values were also studied. The MIC is distinct to the lowest concentration of the compound caused microbial growth inhibition. The serial dilution assay technique of [21] with some adjustments was used. Serial dilutions test were prepared in concentrations ranged from 6.8 to 0.2125 mg/ml for both bacteria and fungi. The extract solution was diluted using nutrient broth (Merck, Germany) for bacteria, and Sabouraud dextrose broth (Merck, Germany) for fungi. 100 µl of the stock extract were transferred to each of the first sterile eppendorph tubes in triplicates containing 100µl of sterile media to prepare the first extract dilution. The remainder of each extract dilution was arranged in the same way. Each extract dilution was inoculated with the selected microorganism. The inocula of bacteria were prepared from 12 h broth cultures and standardized to 10^8 CFU/ml. While, fungal inocula were prepared from 5-7 days fresh fungal cultures, depending on the fungus type, and standardized to 10^5spore/ml. The final volume in each eppendorph tube was 200 µl. The cell-free extract were used as negative controls while the substrate of 6mM $AgNO_3$ (6.8mg/ml) used as positive controls. The growth of bacteria and fungi was determined by absorbance values at 600 nm and 530 nm respectively using UV–Vis spectrophotometer (Spectronic-Milton Roy 1201 UV) [11].

Determination of Minimum bactericidal (MBC) and minimum fungicidal (MFC) Concentrations

To establish minimum bactericidal (MBC) and minimum fungicidal (MFC) concentrations, 0.5 ml AgNPs of each extract and 0.5 ml of sterile distilled water was dispensed, from this test tube labeled '1', 0.5 ml of the mixture was taken and dispensed to a test tube labelled '2' containing 0.5 ml sterile distilled water, this was done twice and from the last test tube labelled '4', 0.5 ml of the mixture was taken so that the mixture remained as 0.5 ml. The stock solution is 0.5 ml (without any dilution) and to this was added 0.5 ml of test organism. To the other tubes containing different concentrations of the extracts 0.5 ml of each test organism was added. Samples were streaked from the tubes onto Nutrient agar and Malt agar extract plates to determine the MBC and MFC, respectively of the extract required to kill the organisms after incubation for 24 h at 37°C, for bacteria, and 3-5 days at 30-35°C, for fungi. These concentrations were indicated by failure of the extract to kill the organisms. The lowest concentration that prevented bacterial and fungal growth after two days of incubation was recorded as minimum bactericidal and/or fungicidal concentrations. The results were compared with Streptomycin and Grisofulvin (Sigma, Germany) where used as standard antibacterial and antifungal agents, respectively [10].

Scanning (SEM) and Transmission (TEM) Electron Microscopy

Scanning and transmission electron microscopic observations were carried out on methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa*, *Klebsiella pneumoniae*, *Alcaligenes faecalis*, *Escherichia coli*, as well as *Candida albicans*, *C. tropicalis, Trichoderma reesei*, *Aspergillus niger,* and *Penicillium marneffei* as an examples for the AgNPs extracts effects on bacterial cells of Gram negative and Gram positive bacteria, as well as yeasts and fungi. One milliliter of 10^8 CFU/ml of bacterial suspensions as well as 10^5 spore/ml of fungi were incubated with the AgNPs extracts at concentration of 6.8 mg/ml for 24 h at 37°C for bacteria and for 5-7 days at 30-35°C for fungi along with control free of AgNPs extract solution. All controls and the treated cells were fixed in 4% glutaraldehyde (Sigma, Germany) and post fixed in 1% osmium tetraoxide (Sigma, Germany) and they were afterward cleaned by 0.1M sodium cacodylate buffer, pH 7.4 (Sigma, Germany). After get rid of the residual osmium tetraoxide, all samples were dehydrated in a graduated acetone (Merck, Germany) series (35 to 100%). The specimens were then dried in the critical dryer and were mounted onto stubs by double-sided carbon tape. Samples were coated with a thin layer of gold by sputter coater, and visualized using high vacuum mode of (JEOL JSM-5500LV) Scanning Electron Microscope (SEM). For the transmission electron microscope (TEM) observation, following the dehydration step, the fixed cells were embedded in Epon and the small blocks of samples were cut with an ultramicrotome (Leica Ultracut-S,). The ultra thin sections were then analyzed at 80 KV using (JEOL 1010) Transmission Electron Microscope.

Statistical Analysis

The agar-well diffusion technique was managed in triplicates. So, the inhibition zone was spoken as average standard deviation (SD). For achieving this purpose, SPSS software version 12.0.0.2 was used.

RESULTS

Antimicrobial Sensitivity Using Agar -Well Diffusion Assay

Inhibition zones as showed in Table (**2**) as qualitative and quantitative effect of AgNPs extracts on tested organisms were measured. The intracellular AgNPs extract of *Verticillium chlamydosporium* showed significant inhibitory effect against *Pseudomonas aeruginosa* (3.2 cm inhibition) and *Aspergillus niger* (2.2 cm inhibition). The mean inhibition zone for them was demonstrating a notable antibacterial impact once put side by side with that of $AgNO_3$ substrate. Interestingly, *Streptococcus epidermidis* was the most sensitive bacterial isolate to all intra and extra cellular AgNPs extracts used. As well as, extracellular AgNPs extract of *Trichoderma viride* and the only extract provided an absolute upshot towards the dramatic bacteria Methicillin-resistant *Staphylococcus aureus* (MRSA), *Salmonella typhimurium* and *Candida tropicalis*.

On the other hand, the intracellular AgNPs extract of the same fungi revealed highly significant effect against broad spectrum of the tested bacteria and fungi, namely; *Staphylococcus aureus, Pseudomonas aeruginosa, Escherichia coli*, *Salmonella typhimurium, Candida albicans* and *Trichosporon sp.* Attractively, the most promising results of the extracellular AgNPs extract of *Trichoderma longibranchiatum* revealed the elite antifungal effect against the most prevalent species in hospitalized patients *Candida albicans, C. tropicalis, Trichosporon sp.* and *A. niger.* Additionally, the intracellular AgNPs extract of the later has a major antibacterial effect on the most frequently isolated member of family Alcaligenaceae in the clinical laboratory, *Alcaligenes faecalis, Trichosporon sp.* and *Geotrichum candidum* the opportunistic fungus causing the majority infection in both immune-competent and immune-compromised patients *Penicillium marneffei.* Also, the Intra and extra cellular AgNPs extract of both *Penicillium aurantiogresium* and *Penicillium roqueforti* have a moderate effect as antimicrobial activity as well as fungal-parasites *Trichoderma reesei* and carbapenemase enzymes-producing *Klebsiella pneumoniae* (KPC) that confer resistant to a broad range of antimicrobial agents.

Minimum Inhibitory Concentrations (MIC)

The MIC for the major investigated bacteria and fungi was recorded in Table (3). MICs ranged from 3.4 to 0.2125 mg/ml. *Pseudomonas aeruginosa, Escherichia Coli, Salmonella typhimurium, Streptococcus epidermidis, Trichoderma reesei* and *Geotrichum candidum* showed maximum MICs (0.2125 mg/ml) while *Enterococcus faecalis, Busillus subtilis* and *Aspergillus niger* showed the minimum MICs (3.4 mg/ml) as corresponding to that of $AgNO_3$ substrate vary from 6.8 to 3.4mg/ml.

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Table 2: Antimicrobial Activity of Mycosynthesized Silver Nanoparticles (AgNPs)

Microbial species	*Culture-Collection Code and source*	Inhibition Zone in Diameter (cm)											
		Myco-synthesized AgNPs extract (mg/ml)										Negative control (microbial cell-free water extract)	Positive control $AgNO_3$ (6mM=6.8mg/ml)
		Intracellular						*Extracellular*					
		*A**	*B**	*C**	*D**	*E**	*F**	*B*	*C*	*D*	*E*		
Bacterial isolates used													
Staphylococcus aureus (MRSA)[#]	*Clinical Isolate*	-	-	-	-	-	-	2.0± 0.3	-	-	-	-	0.2±0.3
Staphylococcus aureus	ATTC25923	2.0± 0.1	2.2± 0.2	1.8± 0.2	1.9± 0.1	1.7± 0.5	1.7± 0.5	1.9± 0.1	1.6± 0.1	1.2± 0.1	2.0± 0.1	-	0.1±0.2
Pseudomonas aeruginosa	ATTC27853	3.2±0.5	3.0± 0.3	1.6± 0.2	1.6± 0.5	1.7± 0.3	1.9± 0.2	1.6± 0.3	1.9± 0.1	1.5± 0.3	1.6± 0.1	-	0.3±0.1
Alcaligenes faecalis	*Clinical Isolate*	1.6±0.2	1.7± 0.5	1.9± 0.2	1.4± 0.5	1.5± 0.1	1.7± 0.1	1.6± 0.5	1.7± 0.2	1.5± 0.1	1.4± 0.2	-	0.2±0.1
Klebsiella pneumoniae	ATTC13885	1.6±0.5	-	1.5± 0.2	-	1.9± 0.2	1.6± 0.1	-	-	1.6± 0.2	1.7± 0.1	-	0.2±0.5
Escherichia coli	ATTC25922	2.0±0.2	2.5± 0.1	-	-	1.5± 0.2	1.7± 0.1	1.2± 0.1	1.2± 0.2	1.2± 0.1	1.2± 0.1	-	0.4±0.1
Streptococcus pyrogenes	ATTC19615	1.5±0.5	1.4± 0.3	1.5± 0.2	1.3± 0.3	1.2± 0.5	1.7± 0.1	1.2± 0.3	1.6± 0.2	1.7± 0.5	1.3± 0.1	-	0.3±0.2
Enterococcus faecalis	ATCC29212	1.4±0.3	1.5± 0.2	1.2± 0.3	1.4± 0.1	1.2± 0.3	1.2± 0.2	1.3± 0.1	1.4± 0.3	1.6± 0.2	1.3± 0.1	-	0.2±0.1
Serratia marscescens	ATTC8100	-	-	-	-	-	1.6± 0.3	1.5± 0.1	1.2± 0.5	-	-	-	0.4±0.1
Busillus subtilis	NRRL B-543	-	-	-	-	1.4± 0.3	1.6± 0.3	-	-	-	-	-	0.3±0.1
Enterobacter cloacae	ATTC23355	1.5±0.3	1.3± 0.2	-	-	-	-	1.7± 0.5	1.4± 0.1	1.4± 0.2	-	-	0.5±0.2
Salmonella typhimurium	ATTC14028	1.7±0.1	2.7± 0.3	1.6± 0.1	1.8± 0.5	1.5± 0.1	2.2± 0.1	2.3± 0.1	1.5± 0.5	1.4± 0.1	1.3± 0.1	-	0.3±0.1
Proteus vulgaris	ATTC13315	1.9±0.2	1.5± 0.5	1.5± 0.1	1.1± 0.3	1.3± 0.1	2.3± 0.2	1.5± 0.3	1.5± 0.1	1.5± 0.3	1.2± 0.2	-	0.2±0.1
Streptococcus epidermidis	ATTC12228	2.3±0.2	3.0± 0.3	2.0± 0.3	2.5± 0.2	2.0± 0.1	1.7± 0.3	2.9± 0.1	2.6± 0.2	1.5± 0.3	2.0± 0.3	-	0.4±0.1
Candida albicans	*ICPCI*	1.6±0.1	2.0± 0.5	1.5± 0.1	1.7± 0.3	2.3± 0.2	1.5± 0.1	1.5± 0.3	2.9± 0.2	1.0± 0.1	2.9± 0.3	-	0.5±0.4
Fungal isolates used													
C. tropicalis	*ICPCI*	1.6±0.1	1.7± 0.1	1.7± 0.1	1.8± 0.1	2.0± 0.5	1.9± 0.1	2.2± 0.2	1.8± 0.1	2.0± 0.1	1.8± 0.5	-	0.3±0.5
Geotrichum candidum	*ICPCI*	1.3±0.1	1.5± 0.3	1.7± 0.2	1.8± 0.1	1.0± 0.3	1.3± 0.3	1.6± 0.2	1.2± 0.3	1.6± 0.1	1.7± 0.5	-	0.2±0.1
Trichoderma reesei	*ICPCI*	-	-	1.7± 0.3	3.2± 0.3	-	-	-	-	-	-	-	0.2±0.3
Trichosporon sp.	*ICPCI*	1.5±0.5	2.0± 0.2	1.9± 0.3	1.8± 0.1	1.4± 0.3	1.6± 0.2	1.6± 0.5	1.9± 0.1	1.5± 0.3	1.8± 0.2	-	0.3±0.2
Aspergillus niger	*ICPCI*	2.2±0.3	0.8± 0.1	1.0± 0.3	1.4± 0.3	1.3± 0.1	1.1± 0.3	1.2± 0.3	2.2± 0.2	1.0± 0.3	1.1± 0.3	-	0.2±0.4
Penicillium marneffei	*ICPCI*	-	1.5± 0.1	1.8± 0.2	1.0± 0.3	1.5± 0.2	1.9± 0.5	-	1.3± 0.5	-	-	-	0.1±0.3
Syncephalastrum racemosum	*Isolate from mummies*	1.0±0.2	1.4± 0.5	1.7± 0.5	1.1± 0.3	1.8± 0.1	1.9± 0.2	1.5± 0.3	1.4± 0.1	1.3± 0.3	1.0± 0.3	-	0.1±0.5

[#]:Methicillin-resistant *Staphylococcus aureus*, "A"*Verticillium chlamydosporium var. chlamydosporium*; "B"*Trichoderma viride*; "C"*T. longibranchiatum*; *"D" Penicillium aurantiogresium;* "E" *P. roqueforti*; "F"*Aspergillus niger*; ICPCI :Immune-compromised ***patients clinical isolate*** *(-) means no growth inhibition zone, ±; standard deviation values.

Minimum bactericidal (MBC) and minimum fungicidal (MFC) Concentrations

The nature of the antibacterial effect of the AgNPs extracts with regard to inhibition/killing of tested bacteria is important. The minimum bactericidal concentration (MBC) ranged from 6.848 against *Serratia marscescens* to 0.265 on *Escherichia coli* mg/ml, while The minimum fungicidal concentration (MFC) was in range 5.986 mg/ml on *Aspergillus niger* to 0.636 mg/ml on *Trichoderma reesei* (Table 3). The MBC: MIC ratio for bacteria or MFC: MIC for fungi is used to specify the nature of the antimicrobial effect against microorganisms used. When the MBC: MIC or MFC: MIC ratio of a pathogen is between 1:1 to 2:1, the chemical substance is considered as bactericidal or fungicidal against that pathogen. On the other hand, if the ratio was > 2:1, the mode of antimicrobial action is more likely to be bacteriostatic or fungistatic. Therefore, MBC: MIC or MFC: MIC ratio was calculated for each bacterial or fungal pathogen, respectively. It was found that the AgNPs extracts exerted a clear bactericidal effect against 8 isolates of bacteria while it exerted bacteriostatic effect against 6 isolates only. For fungi, the extract exerted fungicidal effect against all unicellular yeast fungi used while fungistatic effect was shown against filamentous fungi isolates (Table 3).

Scanning and Transmission Electron Microscope Examinations

Table 3: The MIC, MBC and MFC Values of mycosynthesized silver nanoparticles (AgNPs) against the test microorganisms as compared to the $AgNO_3$ activity.

Microbial specie	**MIC *Concentration* (mg/ml)		MBC *and* MFC *Concentration* (mg/ml)	Antibacterial and/or Antifungal mode	Positive control steriptomycin and/or grisofulvin (mg/ml)	
	AgNPs	($AgNO_3$)			MIC	MBC/MFC
Bacterial isolates used						
Staphylococcus aureus (MRSA)[#]	0.851±0.2	0.8±0.3	2.553±0.3	Bacteriostatic	#	-
Staphylococcus aureus	0.425±0.1	0.3±0.2	1.7±0.2	Bacteriostatic	0.065	0.104
Pseudomonas aeruginosa	0.212±0.1	0.3±0.1	0.424±0.1	Bactericidal	0.04	0.08
Alcaligenes faecalis	1.713±0.2	0.3±0.3	2.569±0.2	Bactericidal	0.085	0.156
Klebsiella pneumoniae	1.721±0.1	0.6±0.2	3.442±0.3	Bactericidal	0.025	0.089
Escherichia coli	0.212±0.3	0.3±0.1	0.265±0.2	Bactericidal	0.047	0.241
Streptococcus pyrogenes	1.732±0.2	0.3±0.3	5.196±0.3	Bactericidal	0.065	0.178
Enterococcus faecalis	3.421±0.3	0.7±0.2	6.842±0.2	Bactericidal	0.098	0.240
Serratia marscescens	1.712±0.1	0.4±0.1	6.848±0.3	Bacteriostatic	0.052	0.102
Busillus subtilis	3.421±0.3	0.3±0.2	5.131±0.2	Bactericidal	0.05	0.096
Enterobacter cloacae	1.712±0.2	0.3±0.3	5.564±0.1	Bacteriostatic	0.045	0.156
Salmonella typhimurium	0.212±0.3	0.3±0.3	0.848±0.3	Bacteriostatic	0.023	0.178
Proteus vulgaris	0.852±0.1	0.8±0.2	1.278±0.2	Bactericidal	0.028	0.250
Streptococcus epidermidis	0.212±0.2	0.6±0.2	0.848±0.2	Bacteriostatic	0.05	0.301
Fungal isolates used						
Candida albicans	0.425±0.3	0.2±0.1	1.275±0.3	Fungistatic	0.045	0.145
C. tropicalis	0.851±0.3	0.3±0.2	2.553±0.1	Fungistatic	0.012	0.210
Trichoderma reesei	0.212±0.2	0.7±0.3	0.636±0.3	Fungistatic	0.047	0.289
Geotrichum candidum	0.212±0.3	0.2±0.3	0.848±0.2	Fungistatic	0.061	0.198
Trichosporon sp.	1.712±0.2	0.3±0.3	5.136±0.3	Fungistatic	0.098	0.147
Aspergillus niger	3.421±0.2	0.8±0.2	5.986±0.1	Fungicidal	0.045	0.265
Penicillium marneffei	0.425±0.3	0.3±0.2	0.850±0.3	Fungicidal	0.026	0.310
Syncephalastrum racemosum	1.712±0.2	0.3±0.1	2.996±0.3	Fungicidal	0.014	0.098

[#]:Methicillin-resistant *Staphylococcus aureus*. **:minimum inhibitory concentration, "A"*Verticillium chlamydosporium var. chlamydosporium*; "B"*Trichoderma viride*; "C"*T. longibranchiatum*; *"D" Penicillium aurantiogresium;* "E" *P. roqueforti*; "F"*Aspergillus niger,* ±; standard deviation values.

General envision of the ultrastructural and morphological interactions between all investigated AgNPs extracts and tested microorganisms using TEM and SEM showed that some AgNPs extracts had variable

behavior concerning morphological and/or ultra-structures features of tested l isolates. Whereas, AgNPs extracts were extremely unusual towards the membrane outlook of some Microorganisms. Amazingly, AgNPs uniformly surrounded some topographic structures and/or ultrastructures of some microorganisms without causing obvious injure or any demolitions to the cells.

While, other AgNPs had a harmful act toward other microorganisms as causing complete cell damaging impairments. The most sensitive bacterial and fungal isolates to mycosynthesized silver nanoparticles (AgNPs) extracts were determinate by antimicrobial assay (Table 4). Additionally, extracellularly produced AgNPs extracts of *Trichoderma viride, T. longibranchiatum, Penicillium aurantiogresium* and *P. roqueforti* as well as intracellular extracts of *Verticillium chlamydosporium var. chlamydosporium* and *A. niger* were tested for their effect on direct contact on morphological and ultrastructure features of *Staphylococcus aureus (MRSA), E. coli, A. niger, Alcaligenes faecalis*, *Candida tropicalis*, *T. reesei*, *C. albicans*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Penicillium marneffei,* respectively using TEM and SEM. (Figures 1 and 2). After exposing tested microorganisms to the mentioned AgNPs extracts, the cells were misshapen and turned out to be gluey, roughly distinguished as a dried cells contrast to control cells (Figures 1 and 2). Furthermore, apart off or severance of the cell membranes of the experienced microbial cells was observed as reaction with AgNPs extracts. Meaning, envision of morphological interaction between AgNPs and *Pseudomonas aeruginosa, Alcaligenes faecalis, Klebsiella pneumoniae, Aspergillus niger* and *Penicillium marneffei* showed that their cells were smashed with a great distraction to their cell walls and cytoplasmic membranes.

Moreover, they entered cells and were seen as intensively black spots within internal organelles. Almost all of AgNPs were aggregated forming colonies with cells biofilm when expelled from the cells just like the case of *Pseudomonas aeruginosa, Alcaligenes faecalis, Klebsiella pneumoniae* and *Penicillium marneffei* at Figure (1). Most cells appeared bordered by discharge of inhomogeneous substance filled with globule of different electron density look. Moreover, some cells were ruined and their cell walls were disorder and the cells were deformed, shrivel,

Table 4: Determination of the most Sensitive Microorganisms to Mycosynthesized AgNPs

Extracellular Biosynthesis of AgNPs Extracts of:		***Most Sensitive Microorganism***	***Intracellular Biosynthesis of AgNPs Extracts of:***		***Most Sensitive Microorganism***
(B)	*Trichoderma viride*	*Staphylococcus aureus (MRSA)*	(B*)	*Trichoderma viride*	*E. coli*
(C)	*T. longibranchiatum*	*A. niger*	(C*)	*T. longibranchiatum*	*Alcaligenes faecalis*
(D)	*Penicillium aurantiogresium*	*Candida tropicalis*	(D*)	*Penicillium aurantiogresium*	*T. reesei*
(E)	*P. roqueforti*	*C. albicans*	(E*)	*P. roqueforti*	*Klebsiella pneumoniae*
			(A*)	*Verticillium chlamydosporium var. chlamydosporium*	*Pseudomonas aeruginosa*
			(F*)	*Aspergillus niger*	*Penicillium marneffei*

"A"*Verticillium chlamydosporium var. chlamydosporium*; "B"*rTichoderma viride*; "C"*T. longibranchiatum*; *"D" Penicillium aurantiogresium;* "E" *P. roqueforti*; "F"*Aspergillus niger*

and a discharged cytoplasm was seen just like the case of *Pseudomonas aeruginosa* and *Klebsiella pneumoniae*. On the other hand, the results of the morphological interaction of AgNPs with MRSA and *C. tropicalis* also showed that AgNPs were aggregated and were stick randomly to their cell walls, showing attraction to the peptidoglycan layer. Figures (1&2) showed that AgNPs produced masses captivated within their surface area, when contacted with *Trichoderma reesei* and *Escherichia coli*. Otherwise, AgNPs entered cells and were seen as rigorous dark electron dense pimples within them, positioned distinctively, stacked to a number of intracellular organelles especially the nuclear chromatin (DNA), but under SEM microscope electron beam, these black spots altered to light spots surrounding the cells. Interestingly, AgNPs that were sited on the brink of DNA almost certainly combined to it composing a novel molecules (AgNPs with DNA) were released from the microbial cells alone without DNA after a while (partly left cells) just in case of *Escherichia coli*, Methicillin-resistant *Staphylococcus aureus* (MRSA)*, Candida tropicalis* and *Candida albicans*.

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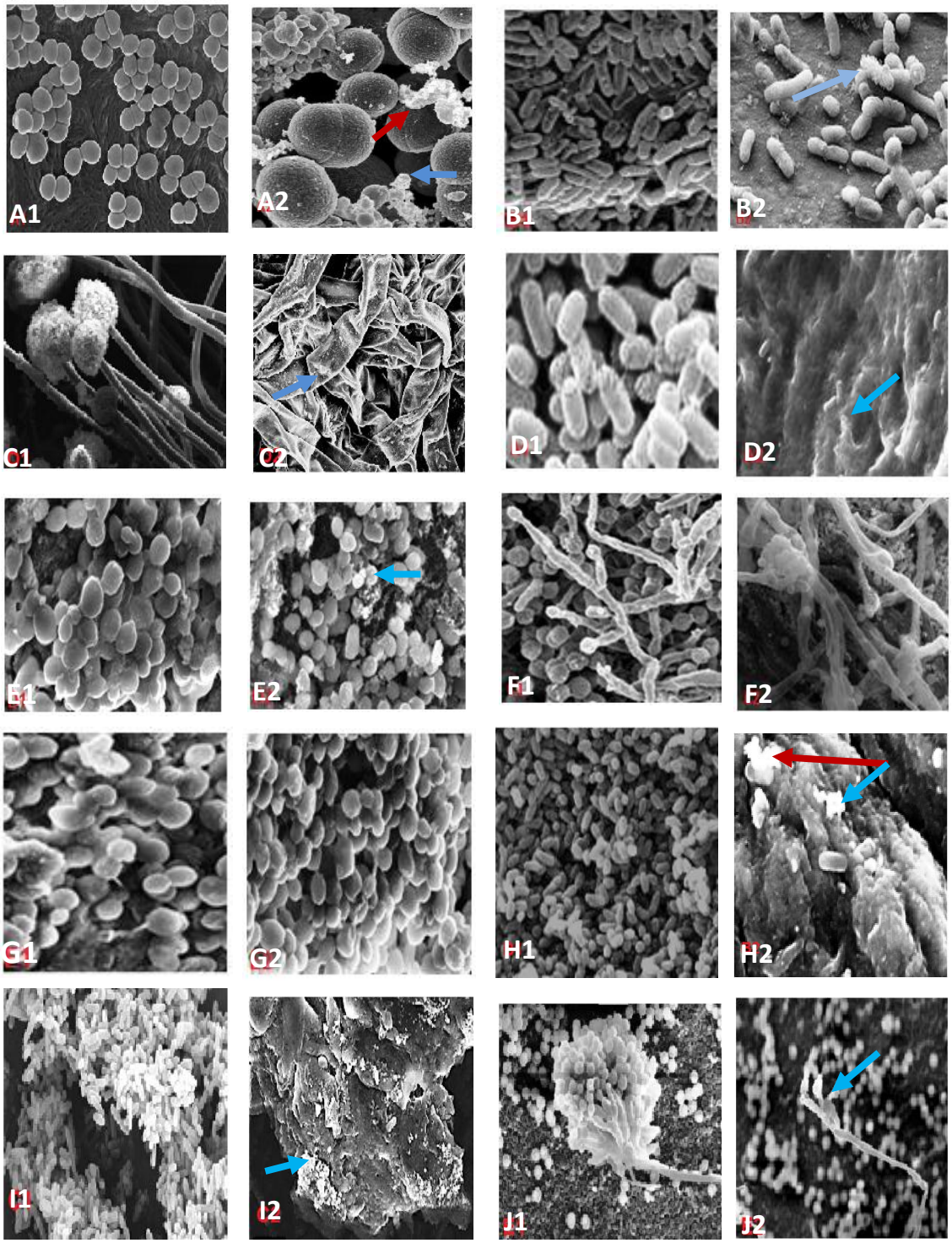

Figure. 1 Scanning Electron Microscope (SEM) Photograph Showed the Effect of Mycosynthesized AgNPs on Morphological Features of Microorganisms. Arrows pointed to nanoparticles (light spots); (A1) Methicillin-resistant *Staphylococcus aureus* (MRSA) control (5um=3200X), (A2) Methicillin-resistant *Staphylococcus aureus* (MRSA) treated with extracellularly mycosynthesized AgNPs extract of *Trichoderma viride* (2um=6700X), (B1) *Escherichia coli* control (5um=3600X), (B2) *Escherichia coli* treated with intracellularly mycosynthesized AgNPs extract of *Trichoderma viride* (2um=6200X), (C1) *Aspergillus niger* control (50um=370X), (C2) *Aspergillus niger* treated with extracellularly mycosynthesized AgNPs extract of *Trichoderma longibranchiatum* (50um370X), (D1) *Alcaligenes faecalis* control (5um=3700X), (D2) *Alcaligenes faecalis* treated with intracellularly mycosynthesized AgNPs extract of *Trichoderma longibranchiatum* (2um=6000X), (E1) *Candida tropicalis* control (5um=3800X), (E2) *Candida tropicalis* treated with extracellularly mycosynthesized AgNPs extract of *Penicillium aurantiogresium* (5um=3700X), (F1) *Trichoderma reesei* control (10um=1600X), (F2) *Trichoderma reesei* treated with intracellularly mycosynthesized AgNPs extract of *Penicillium aurantiogresium* (5um=2700X),((G1) *Candida albicans* control (5um=3500X), (G2) *Candida albicans* treated with extracellularly mycosynthesized AgNPs extract of *Penicillium roqueforti* (5um=3700X), (H1) *Klebsiella pneumoniae* control (5um=4500X), (H2) *Klebsiella pneumoniae* treated with intracellularly mycosynthesized AgNPs extract of *Penicillium roqueforti* (2um=7000X), (I1) *Pseudomonas aeruginosa* control (5um=2600X), (I2) *Pseudomonas aeruginosa* treated with intracellularly mycosynthesized AgNPs extract of *Verticillium chlamydosporium var. chlamydosporium* (2um=3200X), (J1) *Penicillium marneffei* control (10um=1700X), (E2) *Penicillium marneffei* treated with intracellularly mycosynthesized AgNPs extract of *Aspergillus niger* (10um=1600X).

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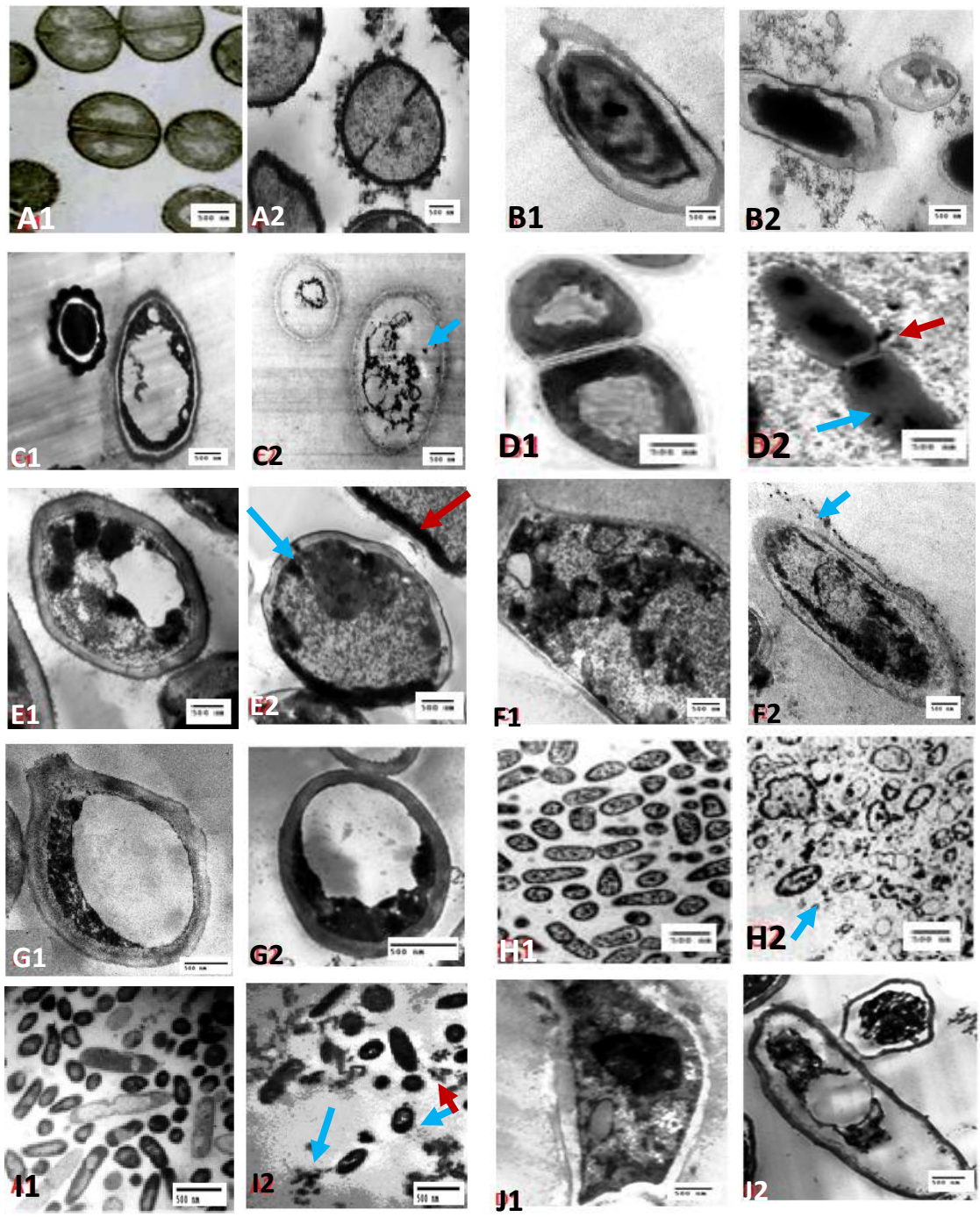

Figure 2. Transmission Electron Microscope (TEM) Photograph showed the effect of mycosynthesized AgNPs on ultra structures of microorganisms. Arrows pointed to nanoparticles (dark spots); (A1) Methicillin-resistant *Staphylococcus aureus* (MRSA) control (30k), (A2) Methicillin-resistant *Staphylococcus aureus* (MRSA) treated with extracellularly mycosynthesized AgNPs extract of *Trichoderma viride* (40k), (B1) *Escherichia coli* control (40k), (B2) *Escherichia coli* treated with intracellularly mycosynthesized AgNPs extract of *Trichoderma viride* (40k), (C1) *Aspergillus niger* control (20k), (C2) *Aspergillus niger* treated with extracellularly mycosynthesized AgNPs extract of *Trichoderma longibranchiatum* (15k), (D1) *Alcaligenes faecalis* control (40k), (D2) *Alcaligenes faecalis* treated with intracellularly mycosynthesized AgNPs extract of *Trichoderma longibranchiatum* (40k), (E1) *Candida tropicalis* control (30k), (E2) *Candida tropicalis* treated with extracellularly mycosynthesized AgNPs extract of *Penicillium aurantiogresium* (30k), (F1) *Trichoderma reesei* control (30k), (F2) *Trichoderma reesei* treated with intracellularly mycosynthesized AgNPs extract of *Penicillium aurantiogresium* (20k) and (40K), (G1) *Candida albicans* control (40k) (G2) *Candida albicans* treated with extracellularly mycosynthesized AgNPs extract of *Penicillium roqueforti* (40k), (H1) *Klebsiella pneumoniae* control (40k), (H2) *Klebsiella pneumoniae* treated with intracellularly mycosynthesized AgNPs extract of *Penicillium roqueforti* (40k), (I1) *Pseudomonas aeruginosa* control (40k) and (I2) *Pseudomonas aeruginosa* treated with intracellularly mycosynthesized AgNPs extract of *Verticillium chlamydosporium var. chlamydosporium* (40k), (J1) *Penicillium marneffei* control (25k), (J2) *Penicillium marneffei* treated with intracellularly mycosynthesized AgNPs extract of *Aspergillus niger* (25k)

DISCUSSION

The prevalent of medicine-resistant microorganisms increase necessitate for novel, economical, efficient, naturally-produced and secure drugs. One of the best candidates to address this need appears to be the natural resources [11].

The consequences of the existing research paper established a number of novel and considerable antimicrobial agents against the tested microorganisms. Variable significant inhibition mechanisms of microbial growth potentially perceived. These regains give the impression of being distinctive on behalf of cell walls dependent-types of the tested microorganisms of the current report when compared with earlier studies running to test the antibacterial activity of several natural substances counting bio-synthesized noble-metal nanoparticles. Chwalibog et al., [22] and Sawosz et al. [23] attributed the variations to the divergence relating Gram negative and Gram positive cell walls which lead Gram negative walls extra challenging to numerous antibacterial agents. These provide evidence that mycosynthesized AgNPs possess various antibacterial machinery making use of diverse mechanisms of action.

The biological impact of nanoparticles on microbial cells is highly dependent on their functional groups present on the surface of nanoparticles-capping protein layer bounding AgNPs that responsible of their stability, which may determine their toxicity versus some microorganisms. Regarding precludes the pressure of the supplementary chemical groups which encircle NPs and to conclude chemical characters of NPs, nano-Ag was biologically synthesized using extra pure double distilled deionized water. Nanoparticles consequent from noble metals were familiar as being nontoxic for the living organisms. Approaches to convey purposeful NP-molecules composites to targeted points inside the cells however hang about to be further considered. In addition, the influence of noble metals' nanoparticles on living cells is peaceful insolently undecided. Taking into consideration physical and chemical properties of AgNPs and biological properties of microorganisms, it is essential to appraise the effect of their auto-aggregation [27].

It was noticed that the outer surface area surrounding both of *Escherichia coli* and *Trichoderma reesei* cells were entrapped with some scattered nano-Ag aggregates or clusters (Figure 2 "[B2 & F2"). The obtained SEM and/or TEM pictures might indicate that this process is potentially initiated by fimbriae network creation enclosing the cells, which probably promotes the development of this biofilm as a protecting shield from any sensed threats; AgNPs in our case . Formation of these biofilm increase the colonization of outer membrane materials, but also protect microbial cells, as assured by the results of [25] which in this case could be a system of protection against nanoparticles toxicity. Sawosz *et al.,* [23] found that the composition of the cell wall of *Listeria monocytogenes* and *Salmonella enteritidis* is fairly distinctive. *Listeria monocytogenes* has a broad (20–80 nm), cell wall constitutes of highly cross-linked layers of peptidoglycan, covalently bound to teichoic acids, whilst the *Salmonella enteritidis* cell wall is slim (5–10 nm) but more complicated; peptidoglycan is bordered by an external membrane be full of lipopolysaccharide and some non- specialized proteins too, that be capable of transfer slighter NPs (around 1 nm). Captivatingly, regardless of major dissimilarities in cell walls structure, the auto-organization among NPs and bacteria cell wall outer layers was influenced by the kind of bacteria to a lesser degree than by the kind of NPs [24]. The formation of nano-Ag clusters reserved the NPs apart of the cells, which might be more protected for the microorganisms. At the same manner, research data of [23] Sawosz *et al.,* revealed that *Listeria monocytogenes* also created biofilm, but above all illustrated the skills to develop congregations with NPs, seeing no solo cells as it was in the case of *Salmonella enteritidis*. Moreover, we noticed that the cell wall and cytoplasmic membrane of *Escherichia coli* and *Trichoderma reesei*, MRSA, *Candida tropicalis*, and *C. albicans* were permeable for nano-Ag, which could be seen as intensively black spots enclosing several healthy organelles within the cells simultaneously (more than one at the same treatment) and not existing in the control cells.

Probably, construction of an AgNPs multi-cells assembly was considered for *Escherichia coli* and *Trichoderma reesei*, MRSA, *Candida tropicalis*, and *C. albicans* as a form of defense mechanism of anti nano-Ag harms. Regarding our findings in the case of *E. coli* and *Trichoderma reesei*, nano-Ag were seen inside unharmed microbial cells without being removed or expelled outside, indicating that these microbial cells are more resistant to nano-Ag. Similar results were observed by [23] Sawosz *et al.,* at the analysis of morphological effects of interaction between nanoparticles and bacteria revealed that nanoparticles entered the cells of *Listeria monocytogenes* but were removed from the cells without any harms to the bacterial cells. While in the case of *Salmonella enteritidis*, nanoparticles were seen inside bacterial cells permanently not expelled, indicating that these cells could be sensitive to AGNPs needed more time of intact to be killed.

CONCLUSION

The findings of the current study unveiled a novel, powerful, and broad spectrum antimicrobial activity of several mycobiosynthesized silver nanoparticle extracts against a large number of human and animal pathogenic microorganisms including drug-resistant bacteria, as well as highly pathogenic fungi. Although none of the studies introduced a definitively proved immediate impact on human health or the environment by silver nonmaterial-containing products, the entirety of the science reviewed suggests some caution and further research are warranted given the already widespread and rapidly growing use of silver nonmaterial. Further investigations on the safety of the mycobiosynthesized silver nanoparticle containing products are required to provide the pharmaceutical companies with economic and effective antimicrobial agents as well as secure carrier-vehicles utilized safely in drug delivery technology.

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