Research Paper

Antifungal Effect of Silver Nanoparticles on Selected Fungi Isolated from Raw and Waste Water

K. M. ALANANBEH, W. J. AL-REFAEE[1] AND Z. AL-QODAH[2]*

Plant Protection Department, Faculty of Agriculture, University of Jordan, Amman, Jordan, [1]Biology Department, Faculty of Science, Taibah University, Madinah, Saudi Arabia, [2]Chemical Engineering Department, Al-Balqa Applied University, Amman, Jordan

In the present study, fungi from waste water of different sources in Madinah, KSA, was isolated in order to determine the effective dose and shape of silver nanoparticles necessary for treating them. Waste water was collected from two sources, homes and hospitals (Ohud Hospital). Additionally, bottled drinking water (Taibah) and autoclaved distilled water was used as control. Uncoated silver nanoparticles were used. The particles were with two shapes (rod, cube) and four concentrations (0, 1, 10 and 100 µg/ml) were selected and distilled water was used as a solvent. Fungi were isolated and purified on potato dextrose agar media. A total of eight genera and nine fungal species were identified: *Aspergillus flavus*, *A. niger*, *A. terreus*, *Fusarium oxysporium*, *F. solani*, *Geotrichum candidum*, *Mucor hiemalis*, *Penicillium chrysogenum*, *Rhizopus oryzae*, *Trichoderma harizianum* and *Trichophyton* sp., *Aspergillus* sp. was the highest in its number collectively, thus was considered for further experiment. Silver nanoparticles were tested on two *Aspergillus* sp. i.e. *A. niger* and *A. terreus*. The gradual growth reduction was clear in both *Aspergillus* species as the concentration of the silver nanoparticles increased. *A. terreus* had higher reduction compared to *A. niger*. No significant differences were found among the 1, 10, and 100 µg/ml concentrations. The rod shaped nanoparticles showed less growth for the fungi studied compared to the cube shaped. It is possible to use silver nanoparticles as antifungal substances; however, more considerations should be taken.

Key words: Silver nanoparticles, fungi, Madinah, *Aspergillus* sp.

Water is considered one of the main components in the development processes in the Kingdom of Saudi Arabia (KSA). Problems associated with water demands include the increase in the number of private and farming wells, uncontrolled pumping, water quality deterioration, uncontrolled agricultural practices, over-irrigation all of which increase soil salinity and leakage from water supply systems[1].

Water resources in the Kingdom include surface water (10-48%), ground water (49-80%), desalinated water (3-6%), and reclaimed water (0-5%)[2]. A combination of growing population and limited potable water resources[3] are reducing the availability and quality of drinking water. In addition, problems resulting from the disposal of waste water continue to appear. Therefore, waste water management practices are vital. Increasing the safe use of recycled water can greatly assist in meeting water requirements, enhance the environment and benefit public health by preserving resources upon which public health protection is based[3].

Water contains soluble and insoluble ingredients such as different salts, gases, plants, and microorganisms. These components are considered impurities in water and their presence may be useful to a certain limit, however, when increased, it may become harmful and preventive measures become needed[4].

Waste water is the water that contains unwanted substances, which affect its quality and thus making it unsuitable for use[5]. Waste water is considered an important source for agricultural use in KSA because of the expected high demand on water in the coming 25 y[6]. Additionally, the population of the Kingdom is expected to reach 45 million after 10 y, and the expected waste water will account for 67% of the total used

***Address for correspondence**
E-mail: z_alqodah@hotmail.com

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water in agriculture. However, the use of waste water has disadvantages. It may cause health problems for human and animals. Contaminates in ground water and waste water includes heavy metals and chemicals that affect not only humans, but also animals and plants[7].

Many microorganisms have been reported in waste water in published studies. The most isolated bacterial genera from waste water were *Escherichia coli*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, and *Acinetobacter radioresistens*[8]. Dematiaceous fungi constituted 63% of the isolated fungi, and included *Cladosporium* (27%), *Phoma* (9%), *Alternaria* (7%), and *Exophiala* (7%)[9]. In another study, the most prevalent genera of fungi were *Penicillium*, *Aspergillus*, *Acremonium*, and *Candida*[10]. Moreover, viruses such as poliovirus, coxsackie virus, rotavirus, echovirus, and hepatitis virus[11], and nematodes *Ascaris* sp., hookworms[12], parasites and protozoa[13] may also exist in waste waters and their existence is considered risky to human beings and requires high attention.

Various methods have been used to resolve water quality problems in natural environments[14,15]. Nowadays, intensive research is conducted to use nanotechnology in water purification[5]. Nanotechnology is used in detecting and removing different pollutants from waste water as a treatment method[16]. When using nanoparticles as adsorbents, nano filtration membranes remove or separate pollutant from water, whereas when using nanoparticles as catalysts for chemical or photochemical oxidation, it affect the destruction of contaminants present[17].

Nanoparticles are one of those methods with great interest due to properties that makes them a good choice for many disciplines[18] such as biology, medicine[19], and much more**.** Gold, silver, copper, magnesium and other materials have been used as nanoparticles against microbes, however, silver was the most efficient[20]. Silver nanoparticles (AgNPs) are currently attracting and increasing attention in many applications including wastewater treatment. These powders have shown unique such as large surface area, quantum confinement and high stability. The most applied method for AgNPs preparation is by the reduction of Ag^+ in aqueous solution. This reduction usually yields colloidal AgNPs with diameters of several nanometres. This colloidal behaviour causes their dispersion in water or organic solvents[21]. Different microbes vary in their response to AgNPs. For example, AgNPs have adverse effect against Gram-negative bacteria compared to Gram-positive due to the peptidoglycan layer thickness and charge[22,23]. The objectives of the current study were to isolate, identify, and quantify the fungal microorganisms in different waste water sources in Madinah and to determine the effective concentration dose and shape of nanoparticles necessary for treating the most prevalent fungus isolated from the different water sources.

MATERIALS AND METHODS

Waste water was collected from two sources in Madinah: homes (raw water, primary treated, secondary treated, tertiary treated) from Water Authority in Al-Kheleel; and hospitals (Ohud Hospital). Additionally, bottled drinking water (Taibah brand from local market) was used based on a preliminary experiment where it showed few microbes among many drinking water sources. Moreover, autoclaved distilled water was used as control. Collection frequency was conducted every month for six months. Five replicates were conducted per water source. Temperature and relative humidity during the collection period was obtained. Temperature ranged from 16-38° and relative humidity was between 0.18-16% during the time of study.

Fungi isolation, purification, and identification:

A 50 µl from each replicate were plated on potato dextrose agar (PDA) media (HiMedia Laboratories Pvt. Ltd), and then incubated at 25° for one week. After that, fungal colonies were purified on PDA and long term stored at -20° for further use. The isolates were identified morphologically using Barnett and Hunter[24].

AgNPs treatment:

Same AgNPs used in Alananbeh *et al*.[25] were used here. Uncoated AgNPs (15±3 nm mean diameter) were purchased from Nano Tech, Egypt and were at least 99.99% pure. Chemical reduction method was used to prepare the particles[26]. A solution of $AgNO_3$ was used as the Ag^+ ion precursor and sodium borohydride was used as a mild reducing and stabilizing agent. The AgNPs solution had grayish yellow color, indicating the reduction of Ag^+ ions to AgNPs. The particles were with two shapes (rod, cube) and had an optical absorption peak at 410 nm, and four concentrations (0, 1, 10, 100 µg/ml) diluted with distilled water were used. Fungal species were selected based on their prevalence in the waste water samples collected.

Treatment assay:

Each fungus was grown for two days on PDA, then a plug (5 mm) of mycelium was cut from the edges of the colony (hyphal tip) and placed on a new PDA Petri dishes. After that 50 µl of the two shapes and the four concentrations of AgNPs were added to the mycelium plug. Plates were incubated at 28° for 10 d. Mycelium growth measurement (diameter) was carried out every 2 d. Three replicates per concentration were conducted and the experiment was repeated twice. The fungal species chosen were studied at the same time. Ag-untreated samples were employed as controls for comparison.

Statistical analysis:

Minitab 17 software was used for data analysis. Analysis of variance (ANOVA) (one way) and descriptive statistics including mean and standard deviation were used to study each factor considered in the collection separately. Means for each factor was grouped based on Tukey's method.

RESULTS AND DISCUSSION

This discussion will consider the main results obtained in this investigation including total cell count, fungi count based on collection date, fungi count based on water source and AgNPs treatment. Fungal isolation was conducted monthly throughout 6 mon. The isolates were counted based on their numbers from the different water sources (Ohud Hospital, raw waste water, primary, secondary, tertiary, Taibah bottled water, and distilled water). A total of 4120 colony/1 ml were counted for the different species of fungi.

Based on the date of water samples collection, ANOVA showed significant differences among dates for fungi (Table 1). CFU of fungi (n=28) means were found to be the highest in January, however, no significant difference was found between January, November and December (Table 2). The CFU were lower and significant in February through April.

Fungal CFU were counted in the different sources of water samples collected. The highest number of fungal colonies was found in Ohud Hospital and raw waste water with a mean of 22 and 26, respectively. No significant differences were found between Ohud, raw and primary water sources (Table 2). There were eight genera and nine species identified (fig. 1). The percent of colonies for each species for the two replicates was as follows: *A. flavus* (2.06%), *A. niger* (22.94%), *A. terreus* (3.88%), *Fusarium oxysporium* (4.25%), *F. solani* (3.28), *Geotrichum candidum* (10.80), *Mucor hiemalis* (1.94%), *P. chrysogenum* (4.37%), *Rhizopus oryzae* (12.26%), *Trichoderma harizianum* (9.71%), and *Trichophyton* sp. (24.51%) (fig. 1). *Aspergillus* sp. was the highest in its number collectively.

The fungus, *A. niger* was found to be the highest isolated either based on its number or based on its occurrence (fig. 2), therefore, it was chosen. Another species, *A. terreus* was also tested in response to AgNPs treatment.

Based on the ANOVA, growth reduction data for fungi studied, the model showed significance at α=0.05. There was no significance between the two experiments and replicates. However, fungus, shape, concentration, day and the interactions between the four factors were all highly significant at α=0.05 (Table 3).

There were differences between the *Aspergillus* sp. using AgNPs for growth reduction. *A. terreus* had higher reduction with a mean of 1.38 cm compared to

TABLE 1: TOTAL FUNGAL CFU BASED ON DATE AND WATER SOURCE

Source	Df[1]	Adj MS[2]	F-value	P-value
Date	5	1179.6	4.03	0.003
Error	66	292.7		
Water source	5	1577.6	6.01	0.000
Error	66	262.5		

[1]Degrees of freedom, [2]adjusted mean square

TABLE 2: MEANS AND GROUPING ANALYSIS FOR THE FUNGAL CFU BASED ON DATE AND WATER SOURCE

Variable		Total fungal CFU	
		Mean[1]	SD[2]
Date	30/11/2013	15.92ab	13.19
	29/12/2012	12.92ab	33.79
	28/01/2013	28.50a	17.60
	26/02/2013	4.92b	8.31
	28/03/2013	3.50b	5.54
	26/04/2013	2.92b	5.50
Water source	Ohud	22.42a	32.29
	Raw	26.50a	15.62
	Primary	15.08ab	15.99
	Secondary	1.83b	2.17
	Taibah	0.08b	0.29
	Tertiary	2.75b	5.31

[1]Means followed with similar letters are not significantly different, [2]standard deviation

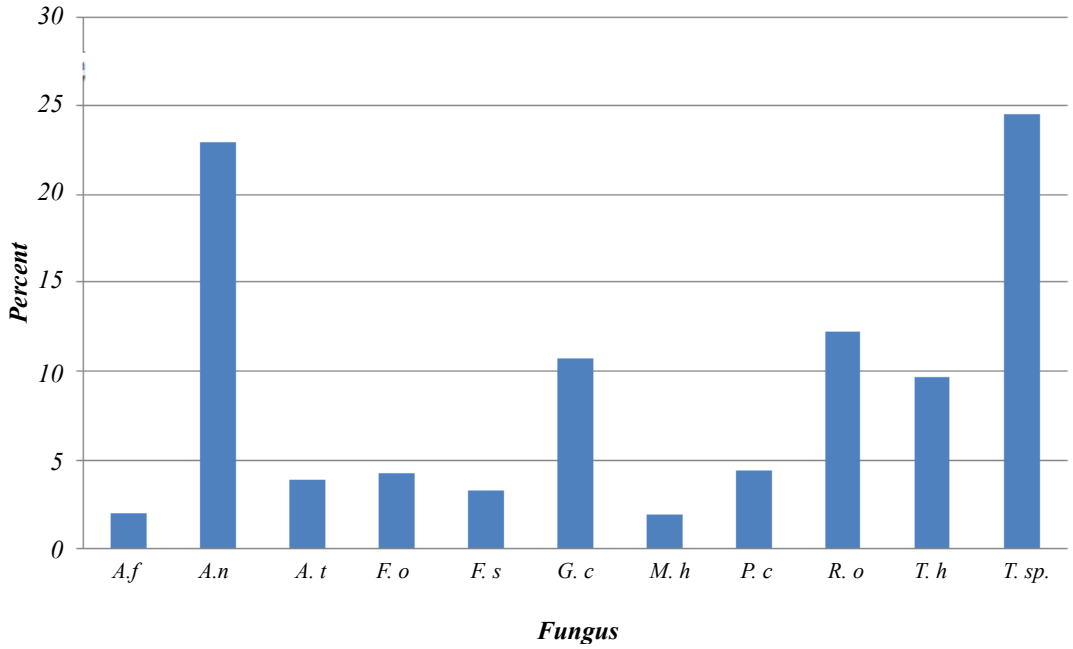

Fig. 1: Percent fungal species identified in this study
***A. f*: *A.* flavus; *A. n*: *A. niger*; *A. t*: *A. terreus*; *F. o*: *Fusarium oxysporium*; *F. s*: *F. solani*; *G. c*: *Geotrichum candidum*; *M. h*: *Mucor hiemalis*; *P. c*: *Penicillium chrysogenum*; *R. o*: *Rhizopus oryzae*; *T. h*: *Trichoderma harizianum*; *T. sp.*: *Trichophyton* sp.**

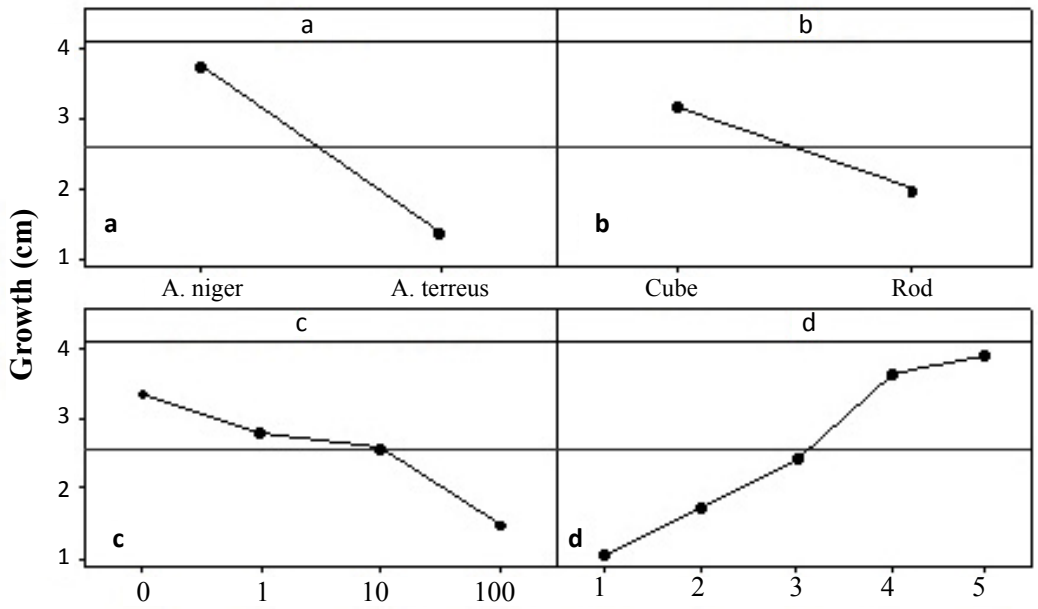

Fig. 2: Growth of *Aspergillus* sp. under different experimental factors
Factors were, fungal species, AgNPs shape, AgNPs concentration, and time for recording mycelium growth (days). (a) Fungus; b. AgNP shape; c. AgNP concentration; d. day

A. niger with a mean of 3.74 cm (fig. 2a). There was difference between the rod and the cube nanoparticle shapes for growth reduction of fungus used. However, rod nanoprticles induced higher reduction with a mean of 1.96 cm compared to cube nanoparticles with a mean of 3.16 cm (fig. 2b).

Based on the nanoparticle concentration, there was gradual growth reduction as the concentration increases, however, no significant differences were found among the 1, 10, and 100 µg/µl concentrations, which had 2.82, 2.58, and 1.49 cm growth reductions, respectively (fig. 2c). There was significant difference between the five days with a mean of 1.06, 1, 75, 2.44, 3.63, and 3.91 cm for day 1, 2, 3, 4, and 5, respectively (fig. 2d).

For the two shapes of AgNPs, there was significant

difference of growth reduction for the two studied fungi. However, the interaction between the rod shape and the *Aspergillus* sp. showed 0.84 and 3.07 cm growth in the case of *A. terreus* and *A. niger*, respectively, compared to the cube×fungus effect, which had a mean growth of 1.91 and 4.41 cm for the *A. terreus* and *A. niger*, respectively (fig. 3).

The gradual growth reduction was clear in both *Aspergillus* species as the concentration of the AgNPs increased. However, the interaction between the *A. terreus* and the 100 and 10 µg/ml concentration showed 0.93 and 1.32 cm growth, respectively, compared to the *A. niger*×concentration effect, which had a growth mean of 2.06 and 3.84 cm for the 100 and

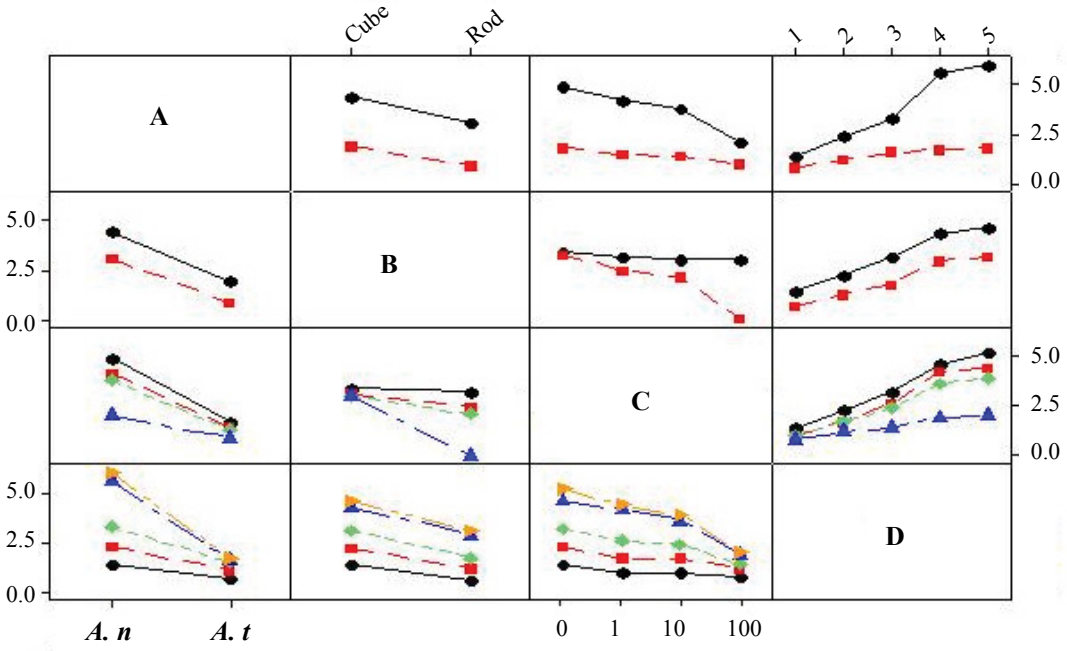

Fig. 3: Interaction of main factors included to analyze effects of different shapes and concentrations of AgNPs against *Aspergillus* sp. A. Fungus: —●— (*A. n*) *A. niger*, —■— (*A. t*) *A. terreus*; B. AgNP shape: : —●— cube, —■— rod; C. AgNP concentration: —●— 0 µg/ml, —■— 1 µg/ml, —♦— 10 µg/ml, —▲— 100 µg/ml; D. Day: —●— day 1, —■— day 2, —♦— day 3, —▲— day 4, —►— day 5

TABLE 3: ANOVA FOR FUNGI MYCELIUM GROWTH INHIBITION DATA MEASURED AS DIAMETER (CM)

Source	DF	Mean square	F	P
Regression	70	32.21	149.63	0.0000
Experiment[a]	1	0.12	0.50	0.4792
Fungus[b]	1	672.84	3125.31	0.0000
Ag NP shape	1	174.73	811.59	0.0000
Ag Np concentration	3	72.99	339.04	0.0000
Day	4	140.86	654.30	0.0000
Replicate	2	0.07	0.32	0.7251
Fungus×AgNP shape	1	2.09	9.72	0.0019
Fungus×AgNp concentration	3	22.86	106.16	0.0000
Fungus×day	4	65.56	304.51	0.0000
AgNP shape×AgNp concentration	3	45.63	211.93	0.0000
AgNP shape×day	4	2.06	9.57	0.0000
AgNp concentration×day	12	5.77	26.79	0.0000
Fungus×AgNP shape×AgNp concentration	3	13.24	61.50	0.0000
Fungus×AgNP shape×day	4	0.37	1.70	0.1500
AgNP shape×AgNp concentration ×day	12	1.72	8.01	0.0000
Fungus×AgNP shape×Ag-Np concentration×day	12	1.30	6.08	0.0000
Error	409	0.22		
Total	479			

[a]Two experiments were conducted at the same time, [b]two fungi were studied: *A. niger, A. terreus*, [c]two shapes were tested: rod and cube, [d]five concentrations were studied: 0, 1, 10, and 100 µg/µl, [e]three replicates were conducted for each concentration, [f]measurements were recorded five times after the fungi were treated (1,3, 5, 7, 9) days

10 µg/ml, respectively (fig. 3). The mycelium growth increased with days in both *Aspergillus* sp., however, the interaction was more in *A. niger* compared to *A. terreus* (fig. 3). The gradual growth reduction was clear in both shapes as the concentration of the nanoparticles increased. The interaction between the rod shape and the 100 and 10 µg/µl concentrations showed 0.00 cm and 2.11 cm growth reduction, respectively, compared to the cube×concentration effect, which had a growth mean of 2.99 and 3.05 cm for the 100 and 10 µg/ml, respectively (fig. 3). The mycelium growth increased with time in both shapes. However, the rod shape nanoparticles had less growth compared to the cubic shape (fig. 3). The whole possible interactions among the main effects (fungus, shape, concentration, days) were all highly significant (fig. 3, Table 3) except the fungus× shape×day interaction.

According to the best of our knowledge, the current study is considered the first for fungal genera and species isolation from waste water sources in the KSA over a period of time. Waste water can be re-used in agriculture and aquaculture. However, the health risks can be major from such practice. There are regulations of using waste water published by WHO in 2006 and also there are local measurements for countries in addition to KSA to monitor the use of waste water.

Different genera of fungi have been isolated from different waste water sources in Madinah. The number and the importance of the isolated microorganisms varied based on the type of waste water from which they were isolated. In April 2013, fungi numbers were more during the first three months then started to decrease. This could be attributed to the temperature at that period. The temperature during the collection period was within the range of 16-38°. In the cold months (November-February), fungal genera *Asprigillus* and *Trichophyton* sp. dominated the isolates, while in warmer months (March-April), *A. niger* was the most dominant microorganism isolated.

There are some studies in the literature reporting some correlation between the time and the type of microorganism. In one study, fungal flora of hospital tap water was evaluated in Iran[27] over a one year period. In that study, different fungal genera were identified: *Aspergillus* was the most recovered genera followed by *Cladosporium* and *Penicillium*. The colony counts varied based on the time of year where *Aspergillus* was more predominated in autumn, *Cladosporium* in winter and spring and *Penicillium* in summer. In another study a total of 340 taxa were isolated from drinking water[28], and filamentous fungi were found more in winter months, while bacteria and yeast were detected in warmer months. In that study, *Penicillium* and *Acremonium* were the most frequently isolated fungi. Drinking water quality is determined by its pathogenic bacteria content. However, the water-borne spores of different fungal genera became potential and more recognized[29].

As mentioned in the above results, the different waste water sources in Medina were used to isolate eight fungal genera and nine species. On the other hand, different fungal genera such as *Phialophora* sp., *Cladosporium* sp., *R. stolonifer*, *Chaetomium* sp., *Alternaria* sp., *Aspergillus* sp., were isolated from tap water in Jeddah city, KSA[28]. This study assured that bottled water has no microbes, whereas two other previous studies reported that bottled samples contaminated with bacteria[30,31].

Some of the isolated microorganisms like *Aspergillus*, *Geotrichum*, *Clostridum*, *E. coli*, *Gardrenella vaginalis* were found in human bodies and considered as natural flora to keep human body functioning normally. However, if their numbers become higher, they might cause sickness[32]. *Aspergillus* sp. may cause Aspergillosis, however certain species like *A. flavus* is 100-fold more virulent than other species and it is a toxin producer[27]. On the other hand, *A. niger* is considered non-pathogenic to human and is widely distributed in nature, however, it can colonize the human body as an opportunistic fungus especially in patients who have immunosuppressive treatment or with severe illness[33]. Similarly, *Fusarium* sp.[34], *Trichodermas* sp.[35], *Penicillium* sp.[36], *Geotrichum* sp.[37] are fungi that infect immunocompromised patients. Moreover, *Mucor* sp. is responsible about mucormycosis and *Rizopus* sp. about mucormycetes infections in humans with immunocompromised and immunocompatent individuals[38].

It should be noted that the number of isolated microbes varied according to the type of water. In this study, Ohud hospital and raw waste waters had the maximum number of fungi compared to the other water sources. Treated water (primary, secondary, and tertiary) also had fungi but less than raw and Ohud hospital waste waters. This was not surprising; hospital waste waters and the raw waste water are rich with different substances such as inorganic like different

metals, nutrients nitrogen and phosphorus, organic matter, and oil and grease. These substances promote microbial growth. Moreover, organisms require small amounts of nutrients to support their growth, thus, even treated waste water may contain enough nutrients for microorganisms (Eutrophication)[39]. Sources of pathogens that were found in hospital waste waters and other water originate from people or animals carriers or infected with a disease and considered a huge risk to public health. Even municipal water and bottled water may contain risk for pathogens[30]. For that reason treating water is important to public health.

AgNPs effect was evaluated with two shapes and four concentrations against different *Aspergillus* species using the Petri dishes method. This method was simple and successful in studying the effect of AgNPs. It was successfully used as in disk diffusion method[40], agar over layer[41], and sol-gel method[42].

Results showed that rod shaped AgNPs at 100 µg/µl produced very high percent inhibition of the test fungi. However, other concentrations also inhibited fungal growth, but not as effectively as the 100 µg/µl concentration. These results suggested that rod AgNPs could be used as antifungal agents. Previous studies reported successful application of different AgNPs shapes and concentrations against fungi such as *Fusarium, Aspergillus* and *Alternaria alternate*, and results showed high inhibition[43]. Moreover, dermatophytes such as *Trichophyton mentagrophytes*[44] and *T. rubrum*[45], were also inhibited by AgNPs, and thus could be considered for clinical applications. AgNPs exhibit high antifungal activity against pathogenic *Candida* sp. at the concentrations of 1 mg/l of AgNPs[46]. Antifungal activity of the AgNPs was comparable with those of ionic silver. However, ionic silver remains cytotoxic at those concentrations that inhibit the growth of the tested yeasts. The AgNPs were found to inhibit growth of the yeasts at very low concentrations that are comparable to those of common antifungals, and they proved that the AgNPs exhibit no cytotoxic effects on human fibroblasts at these concentrations[47].

There are several explanations for the AgNPs mode of action. Metal depletion is one of AgNPs mode of actions that forms irregularly shaped pits in the outer membrane and change membrane permeability, which is caused by progressive release of lipopolysaccharide molecules and membrane proteins[48]. Although their inference involved some sort of binding mechanism that involves interaction between Ag nanoparticles and component(s) of the outer membrane is still unclear. Ag-generated free radicals was reported by using the electron spin resonance (ESR) of Ag nanoparticles[49]. Antimicrobial mechanism of Ag nanoparticles is related to the formation of free radicals and subsequent free radical-induced membrane damage. The antioxidant N-acetylcysteine was used to test whether the antioxidant could influence Ag nanoparticles-induced antimicrobial activity. Other modes of action include DNA condensation, and dehydration of microbial cell was suggested by other authors[50].

Toxicity is a concentration- and size-dependent. Moreover, effectiveness of AgNPs against microbes depends on its shape and size[46,47,51]. The antimicrobial activity varies as AgNPs sizes decrease; it was found that 7 nm was the most effective against bacteria[52] compared to the other sizes because of its small sizes, which can reach the bacterial nuclear content and can be in contact with more surface area[53]. In our study 50 nm was the size for the rod-shaped AgNPs, yet they inhibited the different microbes. This could be explained by the fact that larger AgNPs can persist longer and could serve as continuous Ag ions source[54,55].

This study showed that it is possible to use AgNPs as antifungal substances. AgNPs are considered less harmful, toxic, and cost-effective than other methods. However, in order to get more satisfied results for waste water treatments, future investigations are needed to study other particles against different microbes such as gold nanoparticles, to use smaller sizes of AgNPs to avoid the water characteristics alterations, to study more AgNPs shapes (sphere, triangular, beam) and concentrations, study methods for removing AgNPs from water either by filtration or by using non-pathogenic microorganisms to adsorb them**,** and combine AgNPs with other waste water treatment methods such as UV light, copper ions, or oxidizers, to test the possible synergistic effect.

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Conflict of interest:

The authors declare no conflict of interest.

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