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Green Nano complex as a promising tool for purification of water stations from protozoa and fungi in Sakaka Al-Jouf area

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Due to water-borne pathogenic microorganisms, inadequately treated drinking water is the main cause of preventable sickness and death globally. The presence of fungal strains in potable water has gotten a lot of attention in recent years. Pathogenic microorganisms, including mycotoxigenic fungus, have been discovered in treated drinking water. The goal of this project is to isolate and identify fungus species and protozoa from the water station. In addition, the novel nanoparticles were tested for their ability to kill dangerous microorganisms in water. Four water treatment stations provided forty samples of water. All samples yielded fungal colonies, which were isolated. *Candida* spp. was the most frequent genus (90%), while *Fusarium incarnatum* was the second most common species (70%). *Aspergillus* spp. were found in 62% of the water samples, whereas *Penicillium marneffei* was found in only 12%.

Keywords: Free Living Amoeba; Fungi; Acetaminophen; Nanoparticles

INTRODUCTION

Acetaminophen(N-acetyl-p-aminophenol, paracetamol (Para)) (Fig. 1) is an analgesic and antipyretic medicine with beneficial qualities (Peter et al. 1991, Lee 2017). Numerous attempts to enhance paracetamol's analgesic activity while avoiding hepatotoxicity have been made by altering its structure (Fernando et al. 1980, Harvison et al. 1986, Van de Straatet al. 1986, Harvison et al. 1988). To try to enhance paracetamol's analgesic efficacy via mono-substitution ortho to the hydroxyl group (Harvison et al. 1986). A literature survey indicated that over the last decade, there has been tremendous attention towards studies on metal complex formation using drugs as ligands (Hariprasath et al. 2010). Metal ions have the property of readily losing electrons from their typical elemental or metallic state to generate positively charged ions. Metal-containing compounds have ***several*** benefits over carbon-based molecules when it comes to developing novel therapeutic substances. These metal complexes are found to be interesting due to their biological applications like antifungal, antibacterial, and anti-tumor activity (Farrell et al. 1989). These complexes exhibit a wide range of anticancer activity; they have also been employed as anti-diabetic, antibacterial, and anti-inflammatory agents (Warra 2011).

The primary issue facing the planet is a lack of safe drinking water. Water consumption is boosting the growth of a rapid population increase, yet the amount of available water may be decreasing because of climate alterations. Safe drinking water application is particularly challenging in dry areas. Around 50% of drinkable water in the KSA comes from desalination, 10% from surface water, and 40% from groundwater sources. As a result, the quality and preservation of treated groundwater are critical.

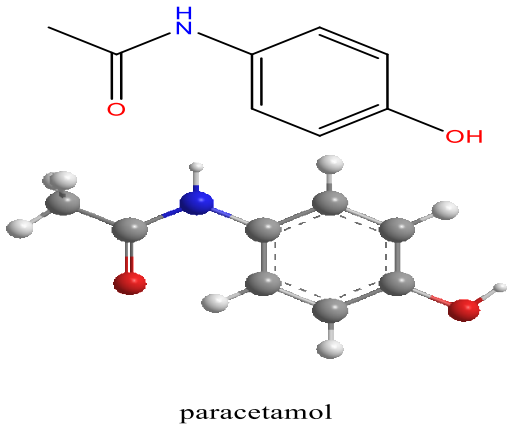
Various bacteria and viruses were found to be thriving in groundwater, and bacterial contamination of groundwater is the source of many dangerous diseases (John and Rose 2005). In addition, fungi are known as drinking water contaminants (Hageskal et al. 2009). Many fungal species found in drinking water are known to cause infectious diseases (Hageskal et al. 2011 & Dutra-de-Oliveira et al. 2013). Recently, opportunistic human fungi have also been isolated from groundwater (Babi et al. 2016 and Oliveira et al. 2016). Fungi were regarded as a

possibly underestimated problem in drinking water (Hageskal et al. 2009, Pereira et al. 2010, Siqueira et al. 2011, Hageskal et al. 2012, Al-gabr et al. 2012, Babi et al. 2017).

The purpose of this research was to detect fungal contamination in treated water and the fungi's potential toxicity to people. We researched four wells in the Jouf region, identifying and assessing the fungus species present in the well water. Additionally, we conducted treatment to determine the feasibility of disinfecting drinking water by eradicating fungal activity using green chemistry. Our goal with this unique approach was to improve awareness of pollution of groundwater supplies by fungi and to analyze the fungi's possible toxicity to people.

Free-living amoebae (FLAs) acceptance in drinkable water constitutes an indirect threat to public health since they may harbor pathogenic bacteria that can bypass water treatment systems and reach the end user (Shawky et al. 2018). The first data demonstrating the distribution of Acanthamoeba in diverse water sources throughout Saudi Arabia's central region reveals that the presence of a high proportion of infectious strains found in recreational water poses a concern to contact lens wearers. Additional research is needed to determine the prevalence of pathogenic Acanthamoeba in Saudi Arabia's varied water sources (Vijayakumar et al. 2018).

The Rotifers form a major portion of the freshwater zooplankton, serve as an important food source for many larger aquatic organisms, and are an integral part of the aquatic food web (Fathibi et al. 2020). Protozoa are unicellular, phagotrophic creatures, and there are free-living freshwater protozoan species in 16 protist groups. They are the major aquatic microbial grazers and the only important grazers in anoxic settings. Ciliates are typically the dominating protozoa in sediments. They produce metabolic wastes that raise the NO2- N content in the water, resulting in a fall in pH. They do, however, aid in the removal of bacteria and detritus from the culture tank (Finlay and Esteban 1989).



paracetamol

Figure1: Paracetamol (Para) drug structure.

MATERIALS AND METHODS

Materials

Acetaminophen (N-acetyl-p-aminophenol (Para), paracetamol), $AgNO_3$, DMF, etc., were acquired from (Aldrich) and utilized with no purification.

Collection of samples

Samples were collected randomly from the Jouf region between January 2021 and March 2021. The 40 samples were collected from four water treatment stations into sterilized polystyrene bottles (500 ml) (Hageskal et al. 2011). For subsequent examination, the samples were kept at +2°C.

Methods

The ^{13}C and ^{1}H NMR spectra (in DMSO-d6) were acquired without using an internal standard on a 600MHZ spectrometer. Thermal tests were conducted in the central laboratory of Jouf University using a thermogravimetric analyzer in a dynamic nitrogen environment heated at a pace of ten ℃ per minute. Molar conductance was determined at ambient temperature, utilizing dimethylformamide (DMF) as the solvent using a JEN WAY 4510 conductivity meter. The analysis of the elements (C, H, N, and S) was carried out utilizing an EL-elemental analyzer**.**

Green chemistry approach for the synthesis of Ag-complexes in nanoscale

To synthesize the silver nano complex, double the level of Para (2 mmol) was mixed with 1mmol of Ag(I) nitrate. At room temperature, the combination was vigorously pounded in an open mortar with a pestle until it melted. After allowing the melted mixture to solidify, it was refrigerated. The material was filtered out and crystallized twice in ethanol, yielding a brown crystal of Ag-nanocomplex.

Isolation of fungi

Ameen et al. (2018) established a technique for collecting fungal species called membrane filtration. Filtration of the water in each sample was performed using sterile cellulose membranes (47 mm; 0.45 µm). These membrane filters were then positioned on rose-bengal potato dextrose agar (rose-bengal-PDA) medium (Hinzelin and Block 1985). Plates were incubated at 25ºC for 6 days and then purified on PDA without rose-bengal.

Identification of fungi

The number of purified fungi was identified and counted. Biochemical characteristics, including carbohydrate assimilation (lactose, maltose, inositol, xylose raffinose, trehalose, galactose, sucrose, melibiose, cellobiose, dextrose, and dulcitol), and nitrogen sources (potassium nitrate and peptone), were applied for yeast identification.

Identification of different filamentous fungi was detected with the aid of macro and microscopic features using an optical microscope (Hoog et al. 2000). Additionally, it was identified using molecular criteria based on sequence analysis of the ITS1-5.8S rRNA–ITS2 region (Animal Health Research Institute, Dokki, Giza, Egypt).

Inhibitory effect of a tested green chemical compound on the mycelial growth of isolated fungi

Under sterile conditions, Petri dishes (9 cm diameter) were supplemented with a sterilized PDA medium. A circular hole of 10 mm at 0.5 mm depth was made (using a special instrument). before adding the tested chemical solution (50, 100 and 150 ppm), each Petri dish was inoculated with two discs of the tested fungus. Negative control was done using P.D. broth media (100 µl) in a well without chemicals, and positive control was done using metayxel (100 µl). Finally, all Petri dishes were at 27 °C for six days in the dark, and the inhibition of the growth of fungal species was measured as inhibition zones (mm).

Concentration, cultivation, and isolation of FLAs:

Separate concentrations of collected water samples (raw or treated) were made using cellulose nitrate membranes (0.45m pore size and 47mm diameter) in a stainless-steel filter holder linked to a suction pump. Filtration was halted immediately before the membrane drying. Following filtering, the membrane was positioned face to face on top of a non-nutrient agar (NNA) plate that had been inoculated with heat-killed *Escherichia coli*. The plate was enveloped in parafilm and incubated at 37 °C to allow free-living amoebae found in water samples to grow rapidly (Federation and Association 2005). For seven days, incubated plates were observed daily using an inverted microscope (Olympus CXK 41, Japan) for the existence of any amoebic growth (Al-Herrawy et al. 2013).

Statistical analyses

Treatment means were calculated using the Waller-Duncan *K*-ratio, t-test when the significant variation of each (P-value < 0.05, P-value < 0.01, P-value < 0.001) was recognized. All of the experiments were repeated twice, and data of one set were the mean of three replications followed by standard deviations.

RESULTS

Paracetamol Ag-nanocomplex

The generated Ag-nano complex afforded the mononuclear metal complex fig. 7. The elemental analysis results indicate that the produced compound has a stoichiometry of 1:2 (metal: ligand) and the molecular formula [Ag(Para)2(NO3)2]. The molar conductance of the Ag-nanocomplex is 7.80 Ohm^{-1} $cm2mol^{-1}$, which is within the range expected for nonelectrolytes.

^{1}H NMR spectra

The spectrum of the free drug exhibits a single peak at 9.44 ppm, which corresponds to an O.H. proton. Furthermore, a protonated signal at 9.86 ppm, as well as multiplet signals at 6.66–7.31 ppm, are assigned to the N.H. proton. By comparing the 1H NMR data of the complex to those of the free ligand, it was established that the spectrum of the complex exhibits a shift of the singlet peak to 8.30 ppm due to the N.H. group, indicating that the ketonic group is involved in the metal atom interaction. Additionally, the complex displayed multiplet signals between 6.80–7.40 ppm, which can be attributed to the aromatic protons, whereas the O.H. protons maintained unaltered (Fig. 2 A, B) (Taha et al. 2020).

^{13}C NMR spectra

The free ligand and the Ag-nano complex ^{13}C NMR spectra were obtained in DMSO-d_6 (Fig. 3). The chemical alterations of all carbon atoms that have been observed are reported in Table 1. By careful comparison, it was indicated from the data that there is no shift in all carbon atoms except for that of the carbonyl group, indicating that participation of the oxygen of the carbonyl group only in complexation (Rehab et al. 2020).

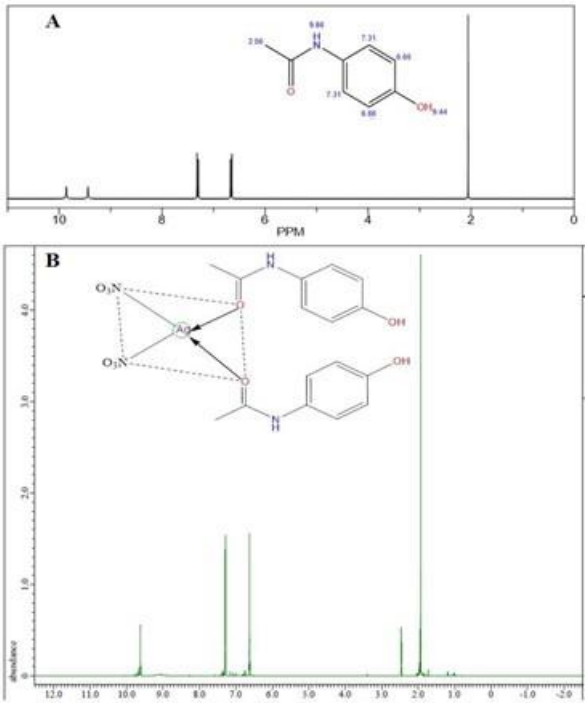


Figure 2: ^{1}H NMR spectrum of (A): The free ligand and (B): The Ag-nano complex.

Conductivity measurements

At room temperature, we determined the Ag-nanocomplex (Λm)'s molar conductivities of 10–3 M solutions. The data obtained indicate that the metal complex is non-ionic (neutral), confirming the coordination

mode of nitrates with the metal cation and implying the complex's nonelectrolyte character.

Within the temperature range of 100–250°C, the first step results in the loss of N2O6, resulting in a mass loss of 23.71 percent (calc. 23.36 percent). The second stage, with an assumed mass loss of 20.24 percent (calc. 20.20 percent), occurs between 260 and 465 °C and results in the loss of C_6H_6ON. Within the temperature varying 465–605 °C, the third stage with an estimated mass loss of 20.27 percent (calc. 20.63 percent) corresponds to the loss of $C_6H_6O_2$. Within the temperature range 605–900 °C, the final stage results in the mass loss of C4H6NO of 15.87 percent (calc. 15.75 percent), leaving Ag metal as a metallic residue. Overall, 79.94 percent of body weight is lost (calc. 79.94 percent).

Table 1: ^{13}C chemical shifts (in ppm) for the free ligand and Ag nano-complex

Carbon	13C chemical shift for the free ligand	13C chemical shift for Ag-nano complex	Assigned to
1	154	153.8	C of benzene ring
2	158.9	169.1	C of carbonyl group
3	24.0	24.29	C of methyl group
4	116	115.7	C of benzene ring
5	123	122.3	C of benzene ring
6	131.1	131.6	C of benzene ring attached to the N.H. group

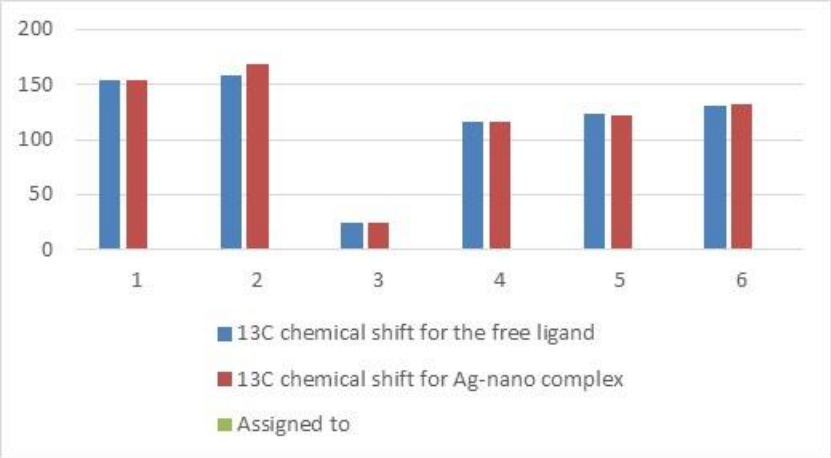


Figure 3: Showing ^{13}C chemical shifts (in ppm) for the free ligand and Ag nano-complex.

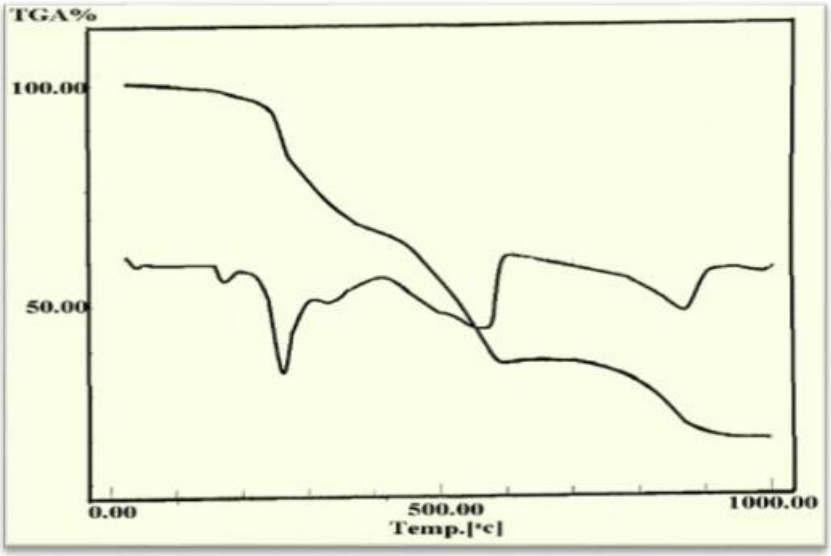


Figure 4: Thermogram of Ag-nanocomplex

SEM images

The nanoparticle's microstructure was studied using SEM techniques, and the related image is shown in fig 5. Depending on this snapshot, the particle size was judged to be between 40-50 nm, with the particles being both spherical and cubic. Although several approaches have been used to generate nanoparticles, this is a rare study on the production of Ag-nano complexes via biosynthesis.

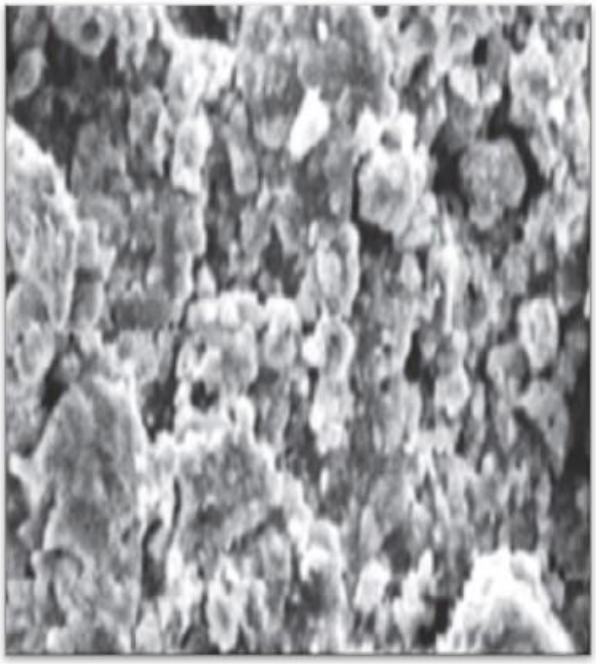


Figure 5: SEM image of Ag-nanocomplex

The sample containing silver nanoparticle's X-ray diffraction (XRD) pattern

The X-ray diffraction (XRD) pattern capable of generating silver nanoparticles was determined to ascertain the crystalline nature of the synthesized Ag-nano complex, and the matching XRD diffractogram could be seen in Fig. (6). The XRD diffractogram was acquired using CuK radiation (= 1.5406), 40 kV- 40mA, 2 scanning modes. The temperature range of 10 to 80 degrees was measured twice. The diffractogram (Fig. 7) was contrasted to the JCPDS standard powder diffraction card, silver file number 04-0783. Four peaks at 2θ degrees 46.6°, 54.55°, 67.88°, and 76.84° in the experimental diffractogram have been recognized as being attributable to silver metal and correspond to the (hkl) values - (111), (200), (220), and (311) planes of silver (JCPDS card number 04-0783). Therefore, the XRD analysis verified that the generated sample contains silver nanoparticles with a face-centered cubic crystal structure (Lee et al. 2013).

The silver nanoparticles' average crystalline size D was determined using the Debye-Scherrer formula, D = 0.9/Cos, where is the wavelength of the X-rays used for diffraction and is the peak's full width at half maximum (FWHM) (Sharma et al. 2014) The FWHM of each of the four peaks was determined by adjusting it using a Gaussian function. The FWHM of the corrected Gaussian curve is used to determine the FWHM of the peak.

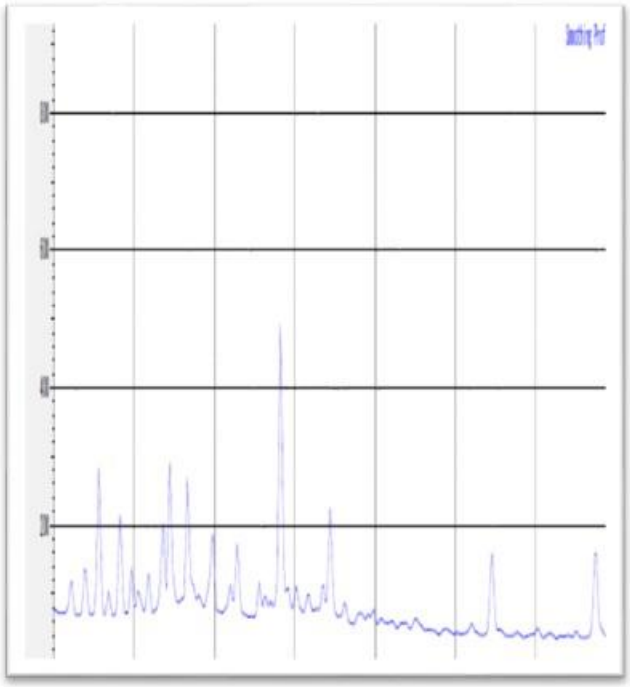


Figure 6: X-ray diffractogram of Paracetamol Ag-nanocomplex

Correlating all of the results acquired for the complex under study provides us with information about the complex's hypothesized structure, which is depicted in Fig.(7).

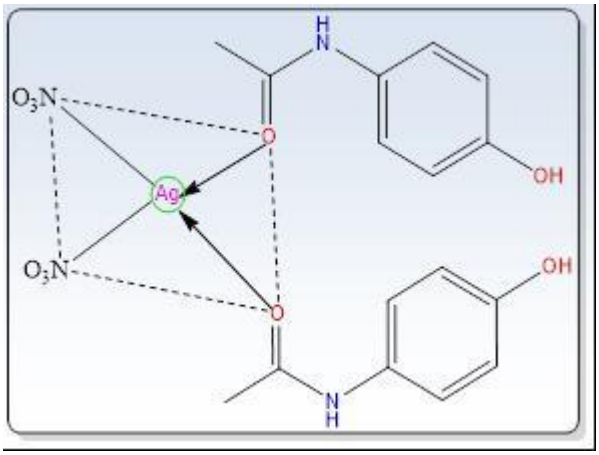


Figure 7: Paracetamol Ag-nanocomplex.

Isolation and identification

In this study, six isolates belonging to four genera have been isolated. Molecular and morphological characterizations were formed (Table 2). The isolates

included two species of *Aspergillus*, two species of *Candida*, one species of both *Penicillium,* and *Fuserium*. *Candida guilliermondii* and *Fusarium incarnatum* were observed in 40 samples from the water treatment stations. *Candida parapsilosis* was found in three water stations. *Aspergillus japonicus* var. *aculeatus* was isolated in two stations, but *Aspergillus penicillioides* and *Penicillium marneffei* were observed only in one station (Table 2). Many studies have proven the presence of fungus in drinking water (Arvanitidou et al. 2000, Warris et al. 2001, Warris et al. 2002, Anaissie et al. 2001, 2002, 2003, Go et al. 2002, Panagopoulou et al. 2002, Kelley et al. 2003, Hapcioglu et al. 2005, Gonc¸alves et al. 2006a, Hageskal et al. 2006, Hageskal et al. 2007a, Hageskal et al. 2007, Kanzler et al. 2007, Kennedy and Williams 2007, Varo et al. 2007, Hageskal et al. 2008, Pires et al. 2008).

A phylogenetic tree was formed (Fig. 8). The detected fungi strains were classified as members belonging to orders Eurotiales, Hypocreales, Saccharomycetales, and family belonging to Trichocomaceae and Debaryomycetaceae (phylum Ascomycota).

The most common genus was *Candida* spp. (90%), which was represented by *Candida guilliermondii* (80%) and *Candida parapsilosis* (10%) (Table 3). *Fusarium incarnatum* was the second dominant species that occurred frequently in 70% of the total isolated fungi. *Aspergillus* spp. were found in 62% and *P. marneffei* in 12 % of the treated water (Table 4).

Table 2: Accession codes for the fungal isolates from the station of water treatment

Fungal species	Accession codes in Genbank
Aspergillus penicillioides	MW957971
Aspergillus japonicus var. *aculeatus*	MW958085
Fuserium incarnatum	MW958219
Penicillium marneffei	MW958224
Candida guilliermondii	MW959195
Candida parapsilosis	MW960416

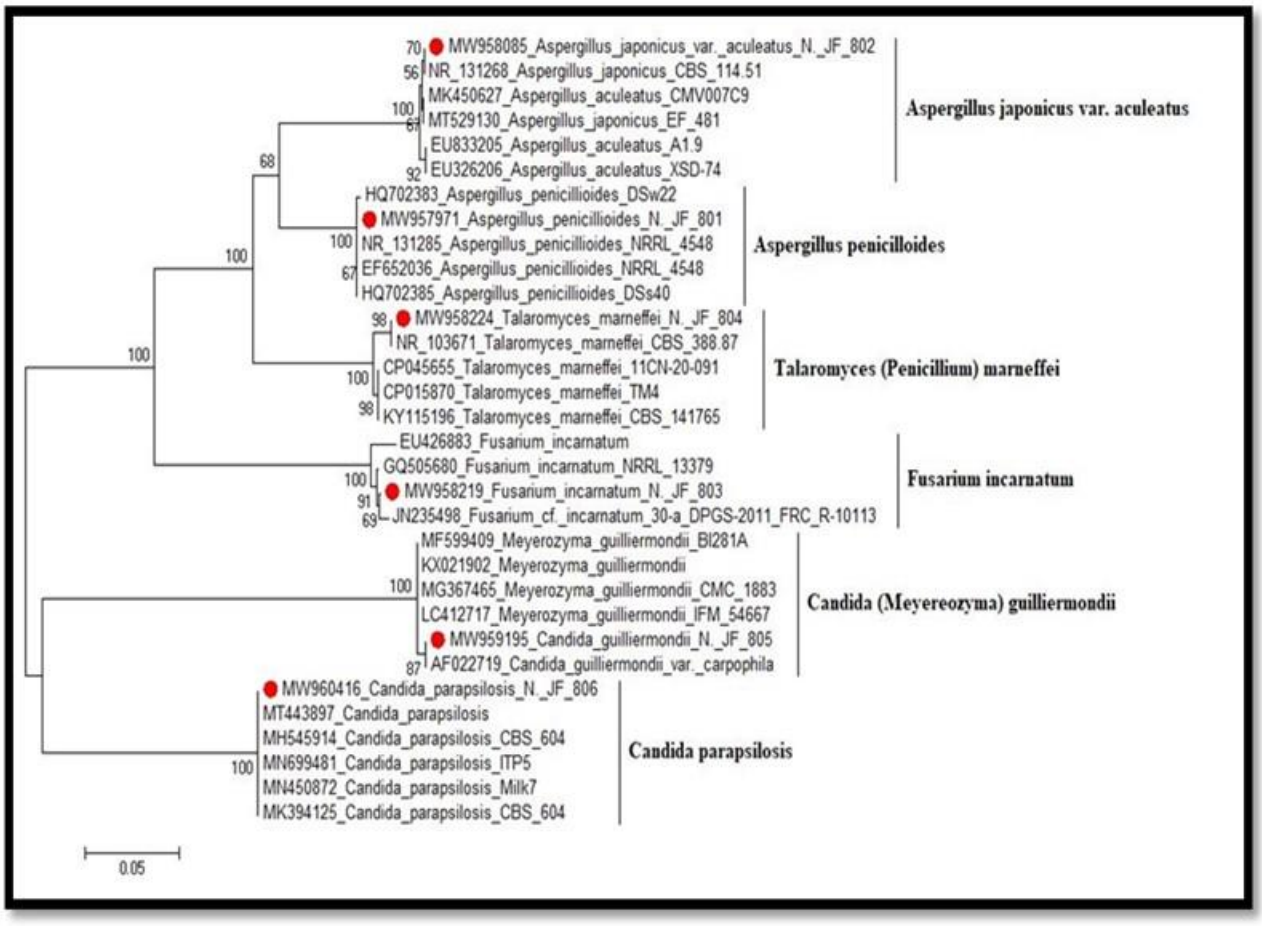


Figure 8: Phylogenetic relatedness of the ITS1-ITS2-5.8S rRNA gene. The maximum-likelihood unrooted tree generated after 500 bootstraps indicated clustering of the tested strains with each related strain.

Table 3: Isolated fungi and their total colony forming units (CFU) in treated water in different stations (by filtration method)

Samples	Fungal species	CFU /ml
W1	*Aspergillus.penicillioides*	39±2.41
	Candida .guilliermondii	8±1.1
	Candida parapsilosis	8±2.1
	Fusarium incarnatum	44±1.6
W2	*Aspergillus.japonicus var. aculeatus*	52±1.5
	Candida guilliermondii	31±2.3
W3	*Fusarium incarnatum*	121±3.
	Aspergillus.japonicus var. Aculeatus	15±3.1
W4	*Penicillium marneffei*	64±1.6
	Fusarium incarnatum	62±3.1
	Candida parapsilosis	6 ± 2.1
	Candida guilliermondii	44±2.8

Table 4: Relative density and colonization frequency of samples after 6-day incubation at 25°C

Fungal species	Relative density (%)	Colonization Frequency (CF%)
Aspergillus	21	62
A. penicillioides	7	15
A. japonicus var. aculeatus	14	47
Candida	42	90
C. guilliermondii	33	80
C. parapsilosis	9	10
Fuserium	27	70
F. incarnatum	27	70
Penicillium	7	12
P. marneffei	7	12

Antifungal assay

Nano bioremediation plays an important role in addressing a variety of environmental problems with inventive and effective solutions. Biosynthetic nanoparticles are being developed for the treatment of various dangerous pollutants using biosynthetic nanoparticles. In addition, it is a cost-effective technology for controlling dangerous pollutants in marine and freshwater, air, and soil. In this study, the antifungal activity of new biosynthetic nanoparticles was evaluated by determining the inhibition percentage of mycelial fungal growth of isolated fungi under study. The bio-synthetic compound presented the greatest biological activity, achieving inhibition of the growth of the species evaluated. However, the biological activity was proportional to the increase in concentration, as shown in Table (5) and Fig. (9). Results appeared to show that all six tested fungi were inhibited by using new synthetic compounds at 50, 100, and 150 ppm. The diameter of inhibition zones was recorded between 40 mm and 49 mm, and at 150 ppm, the highest antifungal effect was recorded. The minimum inhibitory concentrations (MIC) were determined against all isolated fungi with a value of 100 ppm. Kim et al. (2012) were interested in the fungicidal characterization of nano-size silver colloidal solution used as an agent for the antifungal treatment of different plant pathogens. They used silver-nanoparticles at different concentrations (50, 100 and 150 ppm) to determine fungal inhibition percent. Their results show that the most significant inhibition of pathogenic fungi was observed at 100 ppm of AgNPs in PDA media. In addition, Lamsal et al. (2011) reported that the growth fungal inhibition of *Colletotrichum* sp., which is the causal agent of pepper anthracnose, by using AgNP at 100 ppm, whereas concentrations below 50 ppm were enough to obtain the same results as in the field.

Table 5: Mycelium growth of isolated fungi exposed to different concentrations of chemical compound (50, 100, and 150 ppm) for seven days at 27°C in the dark.

Fungal species	50 ppm	100 ppm	150 ppm
A. penicillioides	44±1.24	48±0.3	48±1.24
A. japonicus var. aculeatus	45±1.23	49±0.21	49±1.23
C. guilliermondii	40±0.16	48±0.11	48±0.16
C. parapsilosis	41±1.5	49±0.2	49±1.5
F. incarnatum	47±0.9	47±0.14	47±0.9
P. marneffei	42±0.11	45±0.23	45±0.11

Occurrence of FLAs and other protozoa in drinking water treatment plants

Nagyová et al. (2010**)** photographed morphologically different species of environmental isolates of *Acanthamoeba,* but they did not name them. They had described the mature (resting) cyst previously due to its two walled spheres, which had an outer exocyst running down the cyst's whole length and an inner endocyst interrupted at the ostiole. Within the cytoplasmic margin, a layer of refractile granules was implanted (Pussard and Pons 1977).

Rotifer shows a significant relationship with the chemical and physical factors of the water (Fathibi et al. 2020) (Table 6). Lu et al. (2020) studding warns against making broad generalizations about ciliate function in response to continued global warming, both Positive Acanthamoeba sp. **and** Positive Naegleria sp. recorded an increase in number compared with Positive Rotifers sp. and Positive Ciliates sp (Table 6).

Results appeared that all species of protozoa were inhibited by using a new synthetic compound at 50, 100, and 150ppm. The minimum inhibitory concentrations (MIC) were determined against all isolated protozoa having a value of 100ppm (Fig. 10)

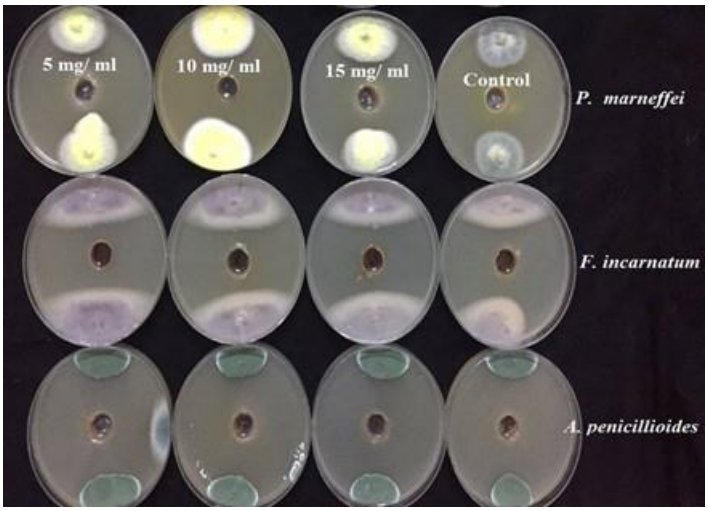


Figure 9: The effect of different concentrations of chemical compound (50, 100 and 150 ppm) on mycelium growth of different isolated fungi for 7 days at 27°C in dark.

Table 6: Occurrence of free-living amoebae and other isolated protozoa in drinking water treatment plants

Place	Examined sample	Positive Rotifers sp.	Positive Ciliates sp.	Positive Acanthamoeba sp.	Positive Naegleria sp.	Positive Nematodes sp.
Gouf	10	6	4	8	8	0
Abrag	10	5	5	8	8	0
Baladya	10	0	0	8	8	0
Alymama	10	6	6	8	8	0
Total	40	17	15	32	32	0
%		53.1	46.8	100	100	0

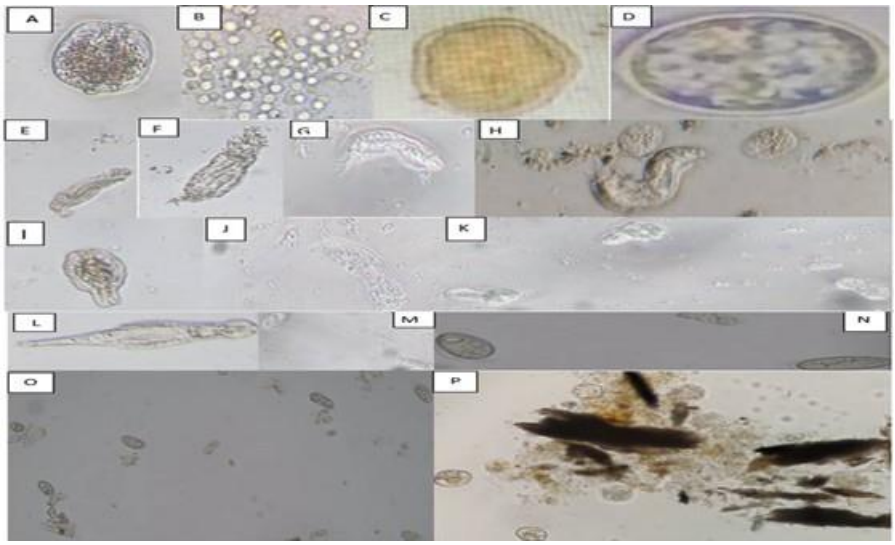


Figure 10: Photomicrograph showed different Free-living amoebae (FLAs) morphology, *Rotifera S.P.,* and *Ciliate SP* A; D; *Rorifera SP* egg, B; *Naeglaria* cyst, C; *Acanthamoeba cyst,* (E; F; G; H; I; J; K;) are *Rorifera SP.*

CONCLUSION

Controlling the pathogenic fungal diseases of vegetables and fruits are very important from an economic point of view. Recently, the greatest effort has been given to developing safety management techniques that demonstrate no risk to humans and animals. The current study reported that AgNPs with a broad spectrum of antimicrobial activity were also effective against pathogenic fungi. *Acanthamoeba* was the most prevalent in the treated water. The presence of potentially pathogenic *Acanthamoeba* species and *Naegleria, Rotifera SP.,* and *Ciliate SP* in drinking water may lead to disorders for users. Moreover, the presence of other free-living amoebae in drinking water exerts an indirect public health hazard as they may harbor pathogenic microorganisms that can escape drinking water treatment processes and reach end-users. Our data clearly showed that FLAs were significantly effective through the drinking water treatment process when selected samples were collected randomly from the Jouf region.

CONFLICT OF INTEREST

The authors declared that present study was performed in absence of any conflict of interest.

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AUTHOR CONTRIBUTIONS

All authors were equally contributed in this work. All authors read and approved the final version.

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