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WOODEN COFFIN BIODETERIORATION ASSESSMENT AND ITS RESTORATION WITH DIFFERENT ANTIMICROBIAL SUBSTANCES

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Abstract

This study aims to isolate and define the microbial infection of a wooden coffin covered with a layer of mud and colored materials and to identify the methods of treating these issues. The polychrome wooden coffin was covered with a ground mud layer, a gesso layer, and a painted layer. Cellulose agar was utilized for the cultivation of fungi, and nutrient agar was utilized for the cultivation of bacteria. The fungi were Fusarium avenaceum, Aspergillus flavus, Fusarium poae, As-pergillus niger, Cladosporium herbarum, and Penicillium Citrinum, while the bacteria were Bacillus jeotgali and G+ve Bacillus Sp. Mixtures of Nano-chitosan with Ag NP, ZnO NP, p-chloro-m-cresol (PCMC), A plant extract of Ceratophyllum Demersum *(CD) with 1, 2, and 3% were selected for the treatment of the microbial infection and the evaluation of the best materials for inhibit-ing microbial growths. Nano-chitosan + ZnO NP, Ag Np (1%) showed the highest fungal inhibi-tion (30mm) against Aspergillus niger. It was observed that Nano-chitosan+PCMC 1% gave the best inhibition for Aspergillus flavus (25mm), Penicillium Citrinum (30mm), and Cladosporium herbarum (43mm). Nano-chitosan+Ag Np (1%) showed the highest fungal inhibition (30mm) against Fusarium avenaceum. The effect equaled 1% for Nano-chitosan+(CD), Ag Np, Nano-chitosan (40mm) against Fusarium poae, while Nano-chitosan+PCMC (1%) showed the highest fungal inhibition (40mm) against Bacillus jeotgali and G+ve Bacillus sp, and Nano-chitosan gave the same effect for G+ve Bacillus sp (40mm)*

***Keywords**: Wooden coffin; Fungi; Bacteria; Microcides; Ag NPs; ZnO NPs.*

Introduction

All wooden artifacts are endangered by insect and microbial attacks [1, 2]. Fungi assume a critical part in the disintegration and deterioration of wooden artifacts due to their tremendous enzymatic activity [3, 4]. Molds can sprout on the outer layer of wood, wooden artifacts, and other organic materials, and deplete carbohydrates and other simple sugars, resulting in chemical and morphological changes of the material structure besides leaving stains [5, 6].

The past decade witnessed the rapid development of Nanomaterials in antimicrobial substances nanoparticles that can abrogate bacterial opposition by coming into direct contact with the bacterial cell wall, without the need to enter the actual cell [7].

p-Chloro-m-cresol (PCMC), which is otherwise called chlorocresol, is utilized as an outside disinfectant and bactericide specialist. It has bactericidal action against gram-positive and gram-negative living beings, alongside yeasts, molds, and spores [8, 9].

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Plants are endowed with a variety of secondary metabolites, which have a powerful antimicrobial activity to treat weak subjects against microbial [10]. A plant extract of *Ceratophyllum Demersum* (CD) is used for the inhibition of mycelial growth [11, 12] and for antimicrobial activity [13-17].

Chitosan is a polymer of D-glucosamine that can be gotten by the deacetylation of chitin [18]. It demonstrated to have fungicidal and fungistatic activity. Nano-chitosan demonstrated to connect with the fungal cell wall and change its structure. Two sorts of components behind the antimicrobial action of the chitosan have been recognized: One of them includes the permeabilization of the plasma membranes of bacteria or fungi due to the electrostatic interactions between amino groups in the chitosan chain and molecules in the cell surface, prompting to the leakage of the intracellular material and the death of the cell [19].

Silver particles have shown significant antibacterial [20, 21] and antifungal properties when used in nanoscale [22-24]. Silver nanoparticles (Ag NPs) are particles with a size of 2-100nm [25]. They alone or in combination with other antifungal compounds could be a better choice in various future applications [26]. Ag NPs misting negatively influence theparameters [27].

ZnO NPs have prevalent antibacterial and antimicrobial properties [28]. Zinc oxide nanoparticles are antibacterial and repress the growth of microorganisms [29-33]. The biological action of nanoparticles relies upon certain variables, including surface interaction, size dispersion, molecule morphology, and molecule reactivity in solution. Therefore, the improvement of nanoparticles with controlled structures that are uniform in size, morphology, and functionality is fundamental for different biomedical applications [30].

The fundamental goal of the current review is to compare the effect of chitosan nanocomposites with the presence of Nano-zinc oxide, Nano silver, *p-chloro-m-cresol* (PCMC), and A plant extract of *Ceratophyllum Demersum* (CD) for the inhibition of microbial infestation. This was attained by unprecedented mixtures of nano-chitosan with Ag NP, ZnO NP, *p-chloro-m-cresol,* and A plant extract of *Ceratophyllum Demersum* (CD) at concentrations of 1, 2, and 3%.

Materials and Methods

Samples were taken from different parts of the coffin (Fig. 1). Four samples were used: Wood, color, surrounding air of the coffin, and ground mud layer.

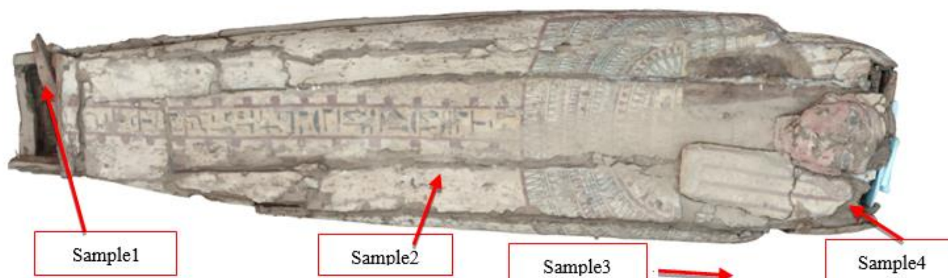

Fig. 1. Locations of microbial swabs: Sample 1 - Wood; Sample 2 – pigment/color; Sample 3 - Surrounding air of the coffin; Sample 4 - Ground mud layer

Cultivation of Swabs

The media of microbial growth (nutrient agar- cellulose agar) were prepared, and the swabs were cultivated on these media and incubated at (28-30°C) for (3-21) days. Then, the microbial growth was examined in preparation for completing the laboratory experi-ments.

Determination of Minimal Inhibitory Concentration (MIC) of antimicrobial agents against the isolated microorganisms

Five microcides were utilized for testing their impact against the confined microorganisms and deciding their MIC. These microcides were Nano-chitosan + Ag Np, Nano-chitosan + ZnO Np, Nano-chitosan + *p*-chloro-m-cresol, Nano-chitosan+ A plant extract of *Ceratophyllum Demersum* (CD), and Nano-chitosan.

Chitosan nanoparticles were prepared [33] by dissolving a 2g of chitosan powder in 2% 100 ml (v/v) of glacial acetic acid to form the chitosan solution. Sodium tripolyphosphate (TPP) (1%w/v) was used as an ionic cross linker. Chitosan nanoparticles were obtained upon the addition of 1mL of TPP into 10mL of chitosan solution under sonication at room temperature for 1 hour using 350 watt sonication doses.

Synthesis of Chitosan Nanocomposites

Solutions of chitosan Nanocomposites were synthesized by using of different amount (0.03, 0.06 and 0.09g) from each of ZnO, Ag, p-chloro-m-cresol, and *Ceratophyllum Demersum* separately into 2% glacial acetic acid in presence of 2g of nano chitosan. The mixture was sonicated for 15 min using ultrasonic sonifier (60MHz time base oscilloscope, Philips, Eindhoven, Netherlands). under dose 350 watt and 2MHz frequency with measurement Suspicion was ±10m/s.

Morphological Analysis of the synthesized Nanocomposites

Transmission electron microscope (TEM) was used to investigate the surface morphology of the synthesized chitosan Nanocomposites. The images of TEM were obtained using a JEM-1230 (JEOL Ltd., Tokyo, Japan) transmission electron microscope under 60kV. In order to prepare the sample to be investigated, it mixed with water for dilution at least 10 times. A drop of sample after dilution putted on 200 mesh copper grids, covered with a carbon membrane and then dried [34] for size and shape morphological investigation [35] presented the obtained TEM images for synthesized nanocomposites, from the figure, it is obvious that, the aggregation of NPs on the chitosan surface increased from Ag to ZnO [36].

One mL of bacterial and fungal isolates suspension was poured onto a nutrient agar and PDA plates respectively. The plates were allowed to dry, then three pores were made by a cork purer in each plate. In dach plate, 100µL of each concentration of the tested microcides were placed in the pore. Plates were incubated at 30°C for 1day compared with control plates (Nano-chitosan). The MIC was determined by measuring the inhibition zone according to the *Method of Brantner* [37].

The light microscopy Carl Zeiss Axio vision order Ks300/4000 Release: 4.72 with analysis unit and digital camera Cam MRc5 was used to identify the fungi and bacteria.

Nu este trimitere la Table 1

Table 1. Experimental materials for resistance to microbial infection

No	Treatment
1	Nano-chitosan + Ag Np 1%
2	Nano-chitosan + Ag Np 2%
3	Nano-chitosan + Ag Np 3%
4	Nano-chitosan + ZnO Np 1%
5	Nano-chitosan + ZnO Np 2%
6	Nano-chitosan + ZnO Np 3%
7	Nano-chitosan + *p*-chloro-m-cresol (PCMC) 1%
8	Nano-chitosan + *p*-chloro-m-cresol (PCMC) 2%
9	Nano-chitosan + *p*-chloro-m-cresol (PCMC) 3%
10	Nano-chitosan + Ceratophyllum Demersum (CD) 1%
11	Nano-chitosan + Ceratophyllum Demersum (CD) 2%
12	Nano-chitosan + Ceratophyllum Demersum (CD) 3%
13	Nano-chitosan 2%

Results and Discussion

The results obtained from the identification of microbial growth are presented in Table 2.

Table 2. The results of the identification of microbial isolates

Swab No	Media used	
	Nutrient agar	**Cellulose agar**
1	*G+ve Bacillus sp.*	*-ve*
2	*Bacillus jeotgali, Fusarium avenaceum, Aspergillus flavus, Fusarium poae*	*Aspergillus niger, Cladosporium herbarum, Penicillium citrinum*
3 air	*-ve*	*Cladosporium herbarum*
4	*-ve*	*-ve*

The molecular characterization of these isolates via PCR, based on the 16S rRNA region [38] showed the largest number of microbial organisms in the layer of colored materials sample. Most of the microbes found on the layer of colored materials sample were *Bacillus jeotgali, Fusarium avenaceum, Aspergillus flavus, Fusarium poae, Aspergillus niger, Cladosporium herbarum, and Penicillium citrinum* (Table 2 and Figs. 1 and 2).

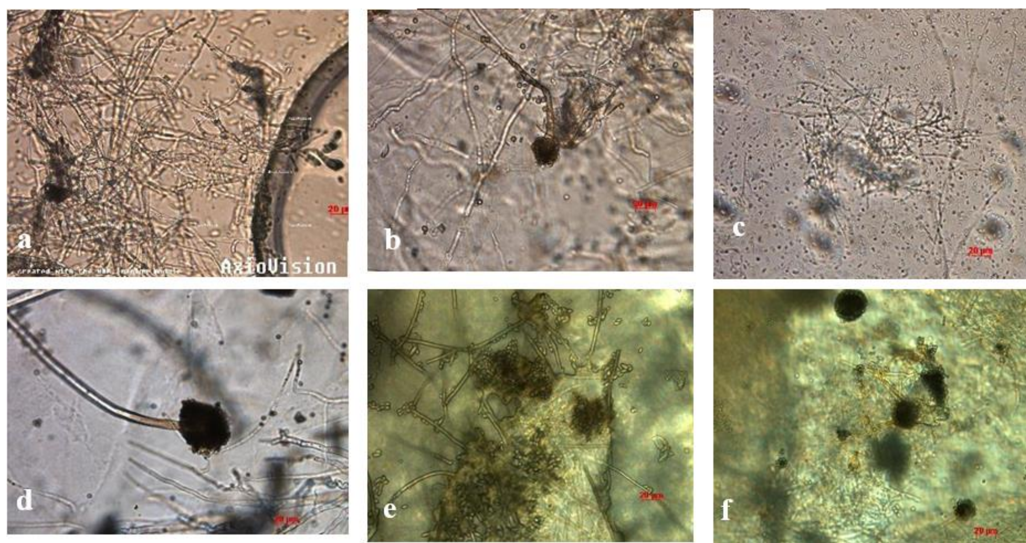

Fig. 2. The fungi isolated from the wooden coffin:
a) *Fusarium avenaceum*; b) *Asp. flavus*; c) *Fusarium poae*; d) *Asp niger*; e) *Clado herbarum*; f) *Pen. Citrinum*

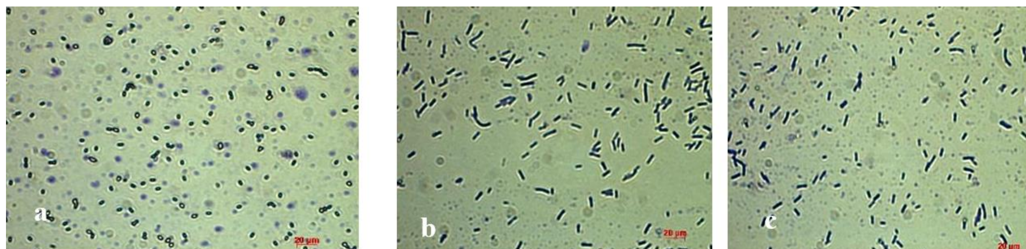

Fig. 3. The bacteria isolated from the wooden coffin:
a) *Bacillus jeotgali*; b) *Bacillus megatrium*; c) *G+ve Bacillus sp.*

This may be due to the thick layer of dust that covered the painted layer because dust is hygroscopic and a site of mold growth [39]. Fungal focuses were up to 100 times upper in the samples containing dust. Moreover, the type of materials played a crucial role in the types of microorganisms and their ability to grow [40]. In contrast, the wood sample did not have any fungal growth but had only one type of bacteria, *G+ve Bacillus sp.* (Fig. 4). The sample of the mud layer did not have any microbial growth. The air sample in the storage contained only one fungi species, i.e., *Cladosporium herbarum*. This mold is also present in the sample of the colored materials.

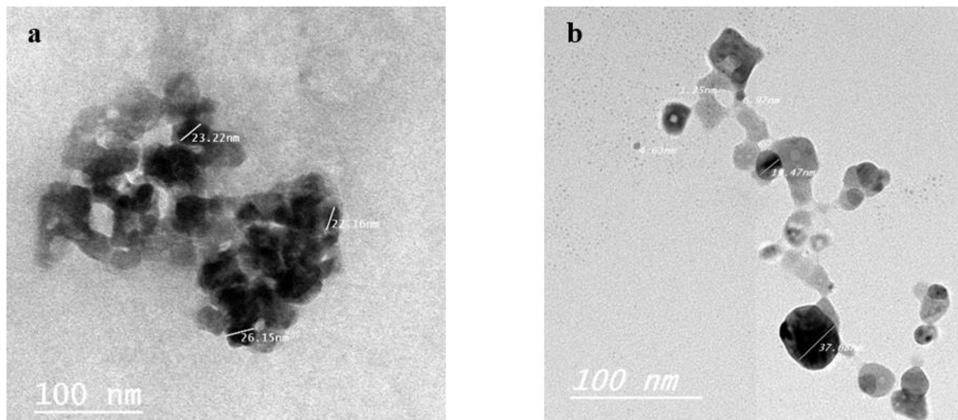

Fig. 4. TEM photographs of the prepared nanomaterials 3% concentration.
(**a**) Nano-Chitosan + Ag NP; (**b**) Nano-Chitosan + ZnO NP

Nanoparticles (NPs) existed on the chitosan surface with uniform distribution and small aggregation (Fig. 4). The dark areas represent the NPs, and the bright areas characterize the chitosan surface. The prepared nanocomposites showed spherical shapes with particle sizes in the range of 23.2nm in the case of Ag NPs and 34nm in the case of ZnO NPs.

According to the MIC values presented in Table 3, some of the experimental materials showed good antibacterial and antifungal activity against the studied microbial strains (Figs. 5 and 6), as follows *Fusarium avenaceum* (Fig**.** 5a) was affected by the materials used in the treatment of microbial infestation. At 1%, the highest effect of Nano-chitosan + Ag Np 30mm was found. It was followed by Nano-chitosan + PCMC and Nano-chitosan + ZnO Np the effect was equal to 25mm, followed by Nano-chitosan, Nano-chitosan, + CD 20mm, and a 2% of Nano-chitosan + Ag Np, Zno Np whose effect was close, followed by Nano-chitosan + PCMC 30mm, then Nano-chitosan +CD 23mm. Nano-chitosan + Ag Np, Zno Np was close to a great extent 50 mm, followed by Nano-chitosan + PCMC. The least effect of Nano-chitosan + CD at 3% was found (Table 3).

Asp. Flavus (Fig**.** 5b) was affected by the materials used for fungal inhibition. At 1%, the highest effect of Nano-chitosan + PCMC 25mm was found. It was followed by Nano-chitosan + ZnO Np 22mm, then the effect of Nano-chitosan + Ag Np and chitosan + CD 20mm. The effect of Nano-chitosan alone was 19mm. The percentage 2% was the highest effect of Nano-chitosan + PCMC 29mm, followed by Chitosan + ZnO Np and Nano-chitosan + CD 24mm. It was followed by Nano-chitosan + Ag Np 23mm, and 3% of Nano-chitosan + PCMC to have an effect of 32mm. It was followed by Chitosan + ZnO Np 30mm, then Chitosan + Ag Np 27mm, followed by Nano-chitosan+ CD 26mm (Table 3).

Fusarium poae (Fig. 5c) was affected by the experimental materials. At 1%, the effect of two substances Nano-chitosan + CD and Chitosan + Ag Np gave the best effect at 40mm. Moreover, Nano-chitosan had the same effect of 40mm as the least effect on the growth of chitosan + PCMC 20mm. The effect of 2 and 3% of Nano-chitosan + CD was equal to Nano-chitosan + Ag Np. It was followed by Nano-chitosan + ZnO Np 40 and 50mm. The least effect was given by Nano-chitosan + PCMC of 30 and 40 mm (Fig. 7).

Asp. Niger fungus (Fig. 5d) was influenced by the materials used for fungal inhibition at 1%, 2%, and 3%. The effect of the two materials was equal Nano-chitosan + ZnO Np. Nano-chitosan + Ag Np of 30, 40 and 50mm gave the highest effect among the materials used, followed by Nano-chitosan + PCMC that had the effect of 21, 26 and 31mm. The least effective material was Nano-chitosan+ CD of 19, 23 and 26mm (Table 3).

Table 3. Determination of Minimum Inhibitory Concentration (MIC) of antimicrobial agents against isolated microorganisms

	The diameter of the clearing zone (mm)												
	Nano-Chitosan +PCMC			**Nano-Chitosan +CD**			**Nano-Chitosan**	**Nano-Chitosan + Ag Np**			**Nano-Chitosan + ZnO Np**		
Name of Microorganism	**1 m/L**	**2 m/L**	**3 m/L**	**1 m/L**	**2 m/L**	**3 m/L**	**2 m/L**	**1 m/L**	**2 m/L**	**3 m/L**	**1 m/L**	**2 m/L**	**3 m/L**
Asp.niger	21	26	31	19	23	25	21	30	40	50	30	40	50
Asp. flavus	25	29	32	20	24	26	19	20	23	27	22	24	30
Penicillium citrinum	30	40	50	24	26	28	25	20	24	30	25	30	40
Cladosporium herbarum	43	46	50	40	49	50	35	20	30	35	0	30	35
Fusarium avenaceum	25	30	35	20	23	28	20	30	38	49	25	40	50
Fusarium poae	20	30	40	40	45	50	40	40	47	50	30	40	50
Bacillus jeotgali	40	46	50	37	44	50	30	25	28	33	30	40	50
G+ve Bacillus sp.	40	43	50	35	40	45	40	0	25	35	28	35	40

The fungus *Cladosporium herbarum* (Fig. 5e) was affected by the materials used to resist microbial infection. The percentages of 1, 2 and 3% of Nano-chitosan + PCMC and Nano-chitosan + CD effect was very close 40:50 mm. They were followed by Nano-chitosan 35 mm, Nano-chitosan+ Ag Np 1% that did not affect the growth. Nano-chitosan + ZnO Np and Nano-chitosan + Ag Np in 2 and 3% was found to have equal effect at 30 and 35 mm (Fig.7).

The fungus *Penicillium citrinum* (Fig. 5f) was affected by the materials used. At 1, 2 and 3% of Nano-chitosan + PCMC that gave the highest fungal inhibition at 30, 40 and 50 mm. It was followed by Nano-chitosan + ZnO Np whose effect was 25, 30 and 40 mm, respectively. It was followed by Nano-chitosan that affected 25mm. Then, the effect of 1% Nano-chitosan + CD 24 mm and 1% of Nano-chitosan + Ag Np 20mm was observed (Table 3).

Bacillus jeotgali bacteria (Fig. 6a) was affected by the substances used for bacterial inhibition. The highest effect was reported for Nano-chitosan + PCMC 1, 2 and 3%; 40, 46 and 50mm followed by Nano-chitosan + CD 37, 44 and 50mm, then Nano-chitosan + ZnO Np, 30, 40 and 50mm. Nano-chitosan + Ag Np was the least effective at 25, 28 and 33mm. The effect of chitosan was only equal to that of Nano-chitosan + ZnO Np 30mm (Fig. 7).

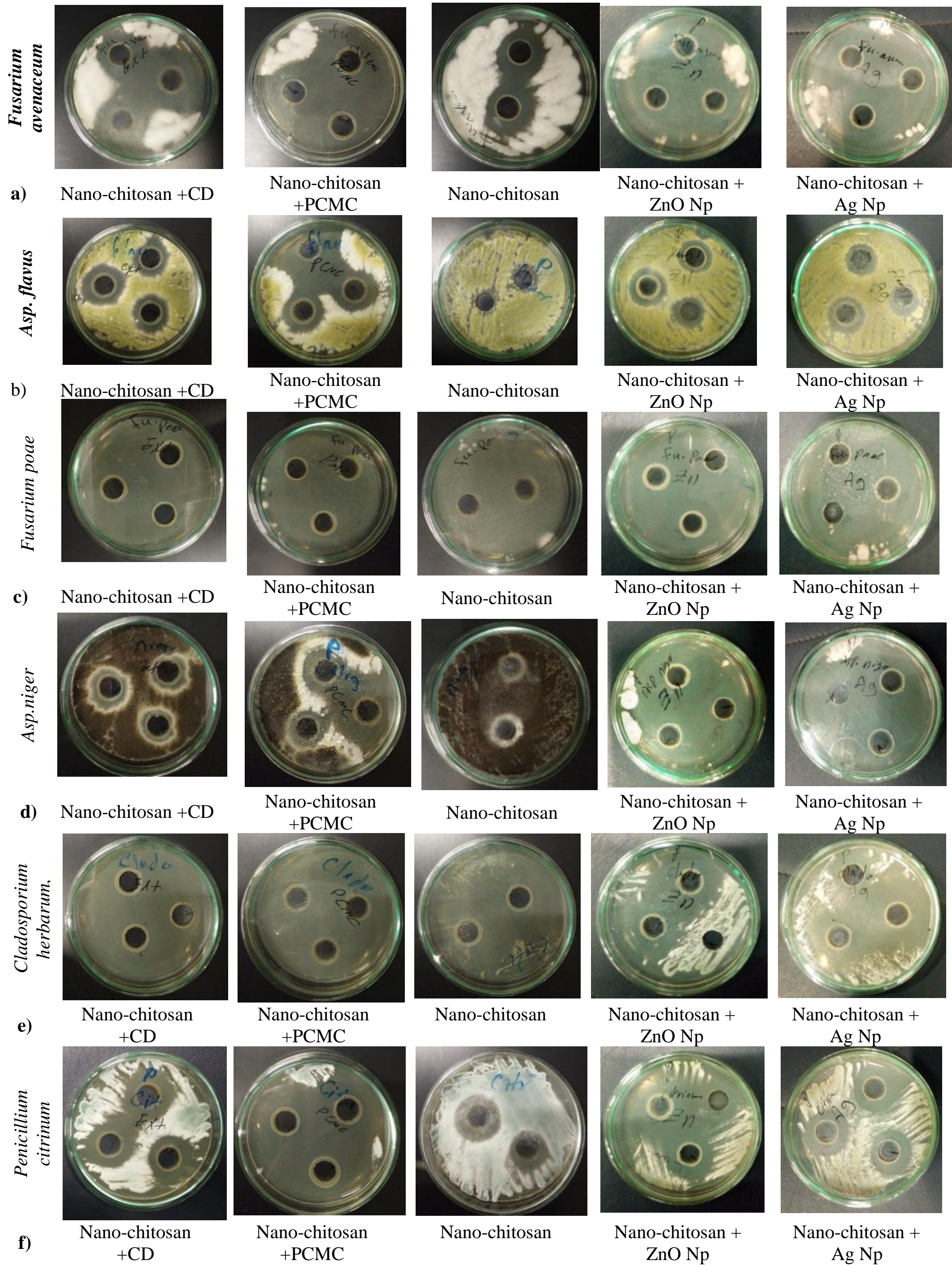

Fig. 5. Antifungal activity of different treatments against the growth of:
a) *Fusarium avenaceum*; b) *Asp. flavus*; c) *Fusarium poae*; d) *Asp. niger; e) Cladosporium herbarum; f) Penicillium citrinum*

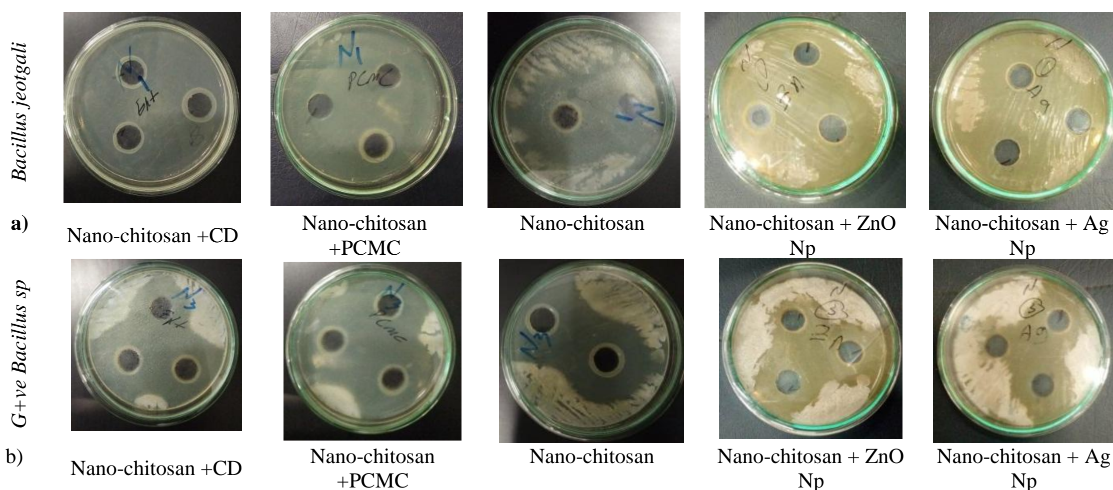

Fig. 6. Antibacterial activity of different treatments against the growth of: a) *Bacillus jeotgali*; b) *G+ve Bacillus sp*.

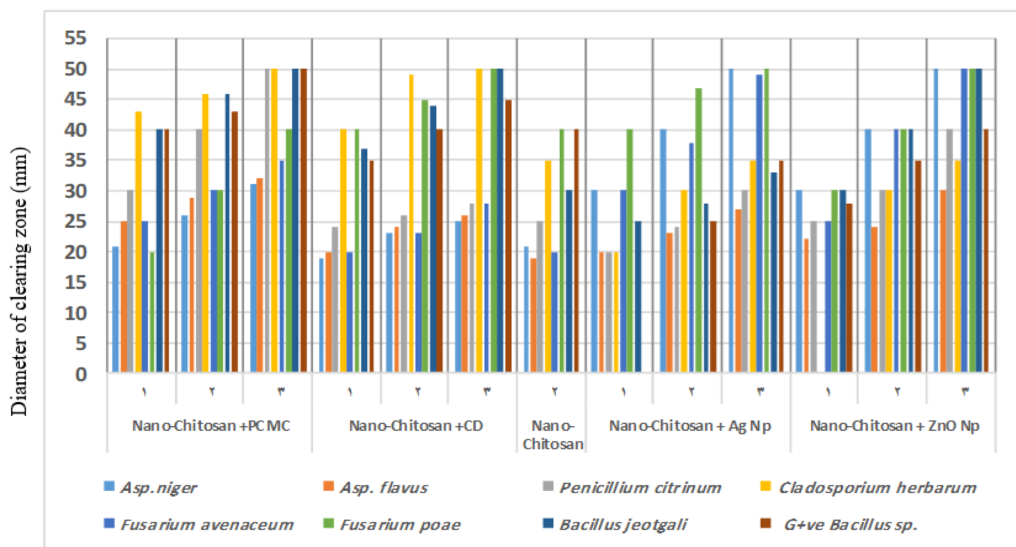

Fig. 7. compares the summary statistics of the effectiveness of the materials used in the treatment of microbial infection of the coffin

G + ve Bacillus sp (Fig. 6b) was affected by Nano-chitosan + PCMC more than other substances in 1, 2 and 3%. Thus, the effect was 40, 43 and 50 mm, respectively, followed by Nano-chitosan + CD of 35, 40 and 45 mm, followed by Nano-chitosan 40mm. Chitosan + ZnO Np gave the best effect at 28, 35 and 40mm, and Nano-chitosan + Ag Np 1% did not affect growth, 2%, 25mm and 3%, 35mm and was the least effective material.

Conclusions

This paper aimed to identify the microbial infestation on the wooden coffin and choosing the best methods for sterilization and treatment. The fungi isolated from the coffin were

Fusarium avenaceum, Asp. flavus, Fusarium poae, Asp.niger, Cladosporium herbarum, Penicillium citrinum, whereas the bacteria were *Bacillus jeotgali, G+ve Bacillus sp*. These microcides were Chitosan + Ag Np, ZnO Np, p-chloro-m-cresol (PCMC), and a plant extract of *Ceratophyllum Demersum* (CD). They were used at a concentration of 1, 2 and 3%. The highest inhibition (25mm) of *Asp. flavus* mycelia growth was observed with the lowest concentration of 1% from nano-chitosan + PCMC. Moreover, Nano-chitosan + Ag Np at 1% gave the best inhibition *of Fusarium avenaceum* (30mm). The highest inhibition of the tested *Fusarium poae* (40 mm) was observed with the lowest concentration (1%) from all the tested materials: Nano-chitosan and Nano-chitosan + Ag Np, PCMC, sh. The highest inhibition of the tested *Asp.niger* (30 mm) was observed with a concentration of (1%) from all the tested materials, Nano-chitosan + Ag Np and Nano-chitosan+ ZnO Np. Also, the highest inhibition of the tested *Cladosporium herbarum* (43mm) was observed with a concentration of (1%) from all the tested materials Nano-chitosan+ PCMC. The highest inhibition of the tested *Penicillium citrinum* (30mm) was observed with a concentration of (1%) from all the tested materials Nano-chitosan+ PCMC, Nano-chitosan+ PCMC at 1% gave the best inhibition (40 mm) for *Bacillus jeotgali, G+ve Bacillus sp*.

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