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Research Article

Detection of Selected Virulence Genes in Escherichia coli and Evaluation of the Antibacterial Efficacy of a Nano-Hybrid Fosfomycin in Urinary Tract Infections.

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Abstract

Escherichia coli are considered as the most common microorganism in pathological samples related to urinary tract infections (UTIs) especially in women and children. They contains many virulence factors helping in pathogenicity. However they are sensitive to fosfomycin that has broad spectrum in its activity against bacteria which its activity also could be confirmed using nanobiotechnology.

This study tried to evaluate the effect of nano-prepared fosfomycin against Escherichia coli isolated from urinary tract infections and compare its efficacy with that of free fosfomycin. The formation of a new complex during the hybridization process was documented by FT-IR, AFM and XRD

Escherichia. coli isolates under study were virulent due to the possessing some of virulence genes such as *afa, pap* and *fimh*. By using nanotechnology, Fosfomycin can be used to prepare nanohybrid antibiotic by loading on Magnesium oxide. The prepared nano-particle was affective against the *E. coli* isolates of UTIs in one third of its concentration Therefore, the hybridization process is economically feasible.

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Introduction

UTIs are almost the common types of bacterial infections, with an estimated 400 million cases and 230,000 deaths globally in 2019. Fifty present of women suffer from this type of infection at least once in their lives, and of course recurrence of the infection is common. The rate of infection increases in the elderly, as in hospitals the highest rate of urinary tract infections is recorded among patients over the age of 65, after urinary system infections [1,2].

Gram-negative bacteria account for the majority of urinary tract infections, although Gram-positive pathogens are also responsible. Uncomplicated UTIs are monobacterial in >95% of cases. The frequent cause of uncomplicated UTIs is *E. coli*, followed by, Klebsiella pneumoniae, Staphylococcus saprophyticus, Enterococcus faecalis, Proteus mirabilis, and group B streptococci [3]. The uropathogenic *Escherichia coli* (UPEC) is considered as the main causative of this type of infection, accounting for more than 75% of uncomplicated and 65% of complex infections in the urinary tract [2]. Several virulence factors are involved in its uropathogenesis, including those that encode proteins of the fimbrial surface adhesins family, such as *Fimh*, Afa and Pap [4]. The *Afa* (afimbrial adhesin) and *Pap* (pyelonephritis-associated pili) operons are the most common adhesion mediators. They facilitate adhesion of *E. coli* to uroepithelial cells and protect them from being washed away by urine flow increasing their pathogenesis [5]. *fimh* (fim stand for of fimbriae), by its specificity for D-mannose-containing structures on host cells, facilitate binding of the fimbriae to host receptors [2].

The excessive and misuse of antibiotics has led to the development of bacteria and the emergence of resistance to antibiotics. Current antibiotics cannot target all bacterial species, so there is a need for new antibiotics that can target metabolic pathways in microorganisms that they cannot modify. Since it is rare to find a new antibiotic, scientists have resorted to developing and modifying methods to get rid of bacteria in the body. Among these methods, we can mention the use of two or more treatments for bacteria that are resistant to multiple treatments or loading them on nanoparticles, which can increase the efficiency of the antibiotic to be delivered to the bacterial cell and eliminate it [6]. Fosfomycin is one of the most important broad-spectrum bactericidal antibiotics used for treatment of UTIs and multidrug-resistant bacterial infection [7,8].

Nanotechnology has recently brought about a global technical revolution through the manufacture of new nanomaterials with advanced physical, chemical and biological properties. Nanoantibiotics are one of those materials that have captured the attention of researchers due to their reduced toxicity and the possibility of using them to inhibit drug-resistant microorganisms, in addition to their low cost compared to traditional antibiotics [9].

Magnesium oxide is one of the important metal oxides due to its unique and excellent properties, including optical, electrical, thermal, mechanical, and chemical properties, as well as its ionic properties and its antimicrobial activity as radical oxygen species (ROS) generation. At the nanoscale level, magnesium oxide shows high effectiveness due to it containing a large number of highly effective edges, the nature of its surface structure and its unnatural cross-linked levels, as well as its surface area-to-volume capacity [10,11].

The current study tried to prepare an efficient nanohybrid-antibiotic using fosfomycin inhibiting virulence factors of UTI causative isolates.

1. Material and methods

1.1. DNA extraction and Gene detection:

Twenty-five uropathogenic *E. coli* isolates were obtained from department of biology/ college of science/ university of Kerbala. Addbio bacterial DNA extraction kits were used for extraction of DNA from the isolates according to the manufacturer instruction (http://addbio.net/default/subc/c03.php?com/b

oard_basic=read/form&com/board/idx=47&&com_board/search/code=&com/board/search/value1=&com/board/search_value2=&com/board/page=&&com/board/id=25&&com/board/id=25). Primers of gene under study were taken from Mishra and Panda [5] study. DNA purity was checked by Nano-drop. Primers sequences for amplification of virulence genes under study are shown in Table (2-1).

Preparation of Fosfomycin nanohybrid:

1) Fosfomycin solution preparation:

Forty ml of suitable solvent was used for dissolve 1.2 gram of Fosfomycin and then 50 ml of same solvent was added to complete the volume to 100 ml.

2) Carrier preparation:

One gram of magnesium-oxide (MgO) was used to preparing the carrier by adding 50 ml of 50% ethanol solution to it, and also, additional amounts of diluted alcohol were added to reach a volume of 100 ml.

Briefly, fifty ml of Fosfomycin solution were added drop by drop to the MgO solution with stirring. The mixture was stirred by magnetic stirrer at room temperature for 24 hrs. and the mixture was placed in an incubator at 40 °C for 18 hrs. The precipitate was separated by centrifuge at 5000 rpm for 20 min, washed with deionized water for several times and was dried at 50 °C. Finally, the dried precipitate was grinded well and gave the symbol Fos-MgO.

Characterization of synthesized Fosfomycin-nanohybrid:

Some methods were used for descripting a nanohybrid Fosfomycin [13,14] as follows:

1) Fourier Transform Infrared Spectroscopy (FT-IR):

The infrared spectrum of both types of fosfomycin nanoparticles, and free particles (each separately), were studied. Also magnesium-oxid (MgO) was studied, by converting it to disks and mix it with potassium bromide after grinding the latter well. The wave-number range of 400-4000 $cm^{-1}$was used in this technique.

The amplification conditions for the virulence genes included one cycle for initial denaturation by 95 °C for 5 min. Denaturation step included 35 sec in 95°. Annealing temperatures for *afa*, *pap* and *fimh* were 55, 57 and 60 °C respectively and for one min for 35 cycle, and extension and final extension steps included one cycle in 72°C for 60 sec and 7 min respectively.

The method of Bashi *et al*. was adopted in [12], to preparing Fosfomycin nanoparticles.

2) X-ray diffraction spectrum (XRD):

By the ability of this technique in explaining of the differences in the layer after and before the process of intercalation, make it easy to characterizing the Fosfomycin-nanohybrid.

3) Atomic force microscope (AFM)

By using AFM current study determined the size, diameter and shape of the aggregation of phosphomycin nanoparticles.

Determination of the inhibitory power of fosfomycin in its free form and nanostructure:

The inhibitory activity of fosfomycin in its free and nano forms was evaluated using *E. coli* of urinary tract infection isolates by the well diffusion method [15], where the concentration of the antibiotic ranged from 16 to 512 µg/ml.

2. Results

Molecular detection of *afa*, *pap* and *fimh* genes.

The results in following images show that the appearance of *afa* bands with a molecular size of 750 bp, *pap* bands with a molecular size of 328 bp and *fimh* bands with a molecular size of 508 bp, in 6 and 10 and 6 of samples respectively. Table 3-1 shows appearance of virulence genes in specimens.

The genes *afa*, *pap* and *fimh* were presented in percentage of (24, 40 and 24 %) respectively which indicate pap is the most predominant gene in the urinary tract

infection isolates under study. (table 3-1). Many virulence factors have a role in the pathogenicity of *Escherichia coli* in urinary tract infection of which adhesion proteins have a prime function. Type 1 fimbriae is encoding result of the *fim* operon cluster and consist of a prime protein, FimA, associated with supplementary proteins *fimG* and *fimF* and the adhesion protein fimh. *pap* gene cluster is an aggregation of 11 genes that encode for main element of the pilus rod. The five defined *afaA* to *afaE* genes encode as known afimbrial adhesions proteins have an important role in the pyelonephritis. *afa* operon encode for proteins act as specific binding actor to human erythrocyte receptors and uroepithelial cells. [16].

Characterization of fosfomycin-nanohybrid

1. Characterization using FT-IR:

Figure 3-4 shows the results of FT-IR spectrum of free Fosfomycin. The stretching vibration of two (OH) groups resulted in the appearance of a broad band at (3182) cm^{-1}. The appearance of the band at frequency of (2939) cm^{-1} could be because to the aliphatic (C–H) stretch. The band appeared at 1633 cm^{-1} related to the stretch of P=O group. In addition, the appearance of the two bands at (1139 and 1076) cm^{-1} indicate to the C - O – C etheric stretch in the epoxide ring [14].

Figure 3-5 shows few bands of absorption in the magnesium oxide (MgO) spectrum of FT-IR. The stretching vibration of the group (O–H) associated with the water physically adsorbed is represented in the band at 3437 cm^{-1} while the surface bending vibration of the (O–H) group of the mentioned water adsorbed appears in the band at 1514 cm^{-1}. Anyhow the asymmetric stretching of group (CO_3^{-2}) represented at the band of 1429 cm^{-1} while the band of (680-580) cm^{-1} represent the metal bond vibration [17].

The combination of MgO carrier and Fosfmycin showed in FT-IR spectrum in Figure 3-6. The broad band at 3414 cm^{-1} which undergoes shift to high frequency is due to the stretch of two hydroxyl groups. The results showed that a shift to P=O stretch group to high frequency as it appeared at 1649 cm^{-1}. Finally, the two bands that belong to stretch of C-O-C in the epoxide ring underwent a shift to low frequencies as they appeared at (1107 and 1003) cm^{-1} [14].

2. Characterization using XRD:

In the present study, the XRD spectrum of MgO alone and Fosfomycin-magnesium oxide were measured to find the differences in layer thickness before and after fosfomycin loading on magnesium oxide layers using Bragg's law [18]. Magnesium-oxide analysis by XRD showed in figure 3-7. As shown, many planes of diffraction are appeared in the spectrum, as follows: At 37.94°, (111) with crystalline distance of 0.23 nm, at 42.94°, (200) with crystalline distance of 0.21 nm, at 62.38°, (220) with crystalline distance of 0.14 nm, at 74.74°, (311) with crystalline distance of 0.126 nm and at 78.66° the diffraction plane was (222) with crystalline distance of 0.121 nm [17]. Figure 3-8 shows the XRD analysis after the intercalation between Fosfomycin and MgO layers as follows: crystalline distance of 0.49 nm was for diffraction plane (003) which showed at 17.95° while crystalline distance of 0.23 nm showed for another plane (006) at 37.56°. The XRD results provided a convenient indication for the intercalation between Fosfomycin and MgO to compose Fos-MgO.

3. Characterization using AFM

The outer surface of Fos-MgO was scanned by atomic force microscope (AFM). Figure 3-9 show the three-dimensional section of prepared nano antibiotic. The nanoparticles were almost spherical in shape with height of 232 nm. Figure 3-10 showed the chart of granularity cumulation distribution of Fos-MgO hybrid. Nanoparticles with highest percentage (11.65%) was belonged to diameters of (60 and 70) nm. Fos-MgO

hybrid showed particle size means about of 84.3 nm. These results were different from those obtained by Jabur [11] who obtained a diameter average size of 77 nm for the prepared nanohybrid ciprofloxacin loaded on MgO.

4. The inhibitory efficacy of the antibiotic-nanohybrid on *E. coli*:

The inhibition activity of free and the nano-fosfomycin antibiotic (Fos-MgO) was tested on ten of *E. coli* isolates of UTI. Table 3-2 shows the inhibition activity of free Fosfomycin. The diameters size indicates increasing of inhibition by increasing of concentrations. Using 512 µg/ml of Fosfomycin the diameter size of inhibition varied from 22 to 26.6 mm for *E. coli* 6 and *E. coli* 1 respectively. On the other hand, table 3-2 show that Fos-MgO nanohybrid with concentration of 512 µg/ml gave large inhibition diameter of 26.5 mm on *E. coli* 1 isolate and small inhibition diameter of 22 mm on *E. coli* 6 isolate.

5. Discussion and Conclusion

Many studies applied to comparing between the inhibitory activity of free antibiotics and that of nanohybrid one against pathogenic bacteria. In a study conducted by Al-Fatlawi [19] demonstrated that the nanoparticle laded antibiotic was very strong in inhibiting of isolated microbes. However, the study investigated the effect of azithromycin of *Klebsiella pneumonia* isolates. Furthermore, the current results were similar to those announced by Kumar *et al.* [20] where found the high efficiency of doxycycline nanoparticle in the inhibiting of *E. coli* isolates compared to that of free doxycycline.

Fosfomycin is an antibiotic with a broad-spectrum activity and inhibit cell wall synthesis by interfering with the formation of UDP N-acetylmuramic acid (the peptidoglycan precursor) [21,22] Also Nano-particles themselves have unique chemical and physical properties that can overcome the mechanisms that bacteria use to resist antibiotics, and thus they will play a role in activity against microbes in addition to antibiotics. For example, Nano-particles can bind to the microbial membrane and disrupt it, thus causing the leakage of cytoplasmic components to the outside, or implemented through the membrane into the cell and bind to intracellular components such as enzymes, ribosomes and nucleic acids, thus stopping the natural cellular mechanisms [23]. Nano-particles can also cause oxidative stress and electrolyte imbalance, thus causing enzyme inhibition and thus the death of the bacterial cell [24].

Referring to the inhibitory activity tables (3-2) and (3-3), it is generally clear that the inhibitory activity of the nano-antibiotic is lower than that of the free antibiotic. However, the nano-antibiotic is still more efficient than the free antibiotic in inhibiting bacteria for several reasons, Firstly, the most important of which is that the nano-antibiotic consists of two parts (antibiotic) + carrier (and if we take into consideration the cost of each of the antibiotic and carrier separately, this means that the nano-antibiotic is less expensive than the free antibiotic. Secondly, nano-antibiotics are more effective than free if they are compared using the same concentration. Thirdly, nano-antibiotics improve the pharmacokinetics of the drug, which includes its metabolism, distribution, absorption and excretion. This type of antibiotic improves the therapeutic index of the drug, increases its solubility in the serum, and prolongs its circulation period in the blood. Fourthly, it controls its release into tissues and cells [25].

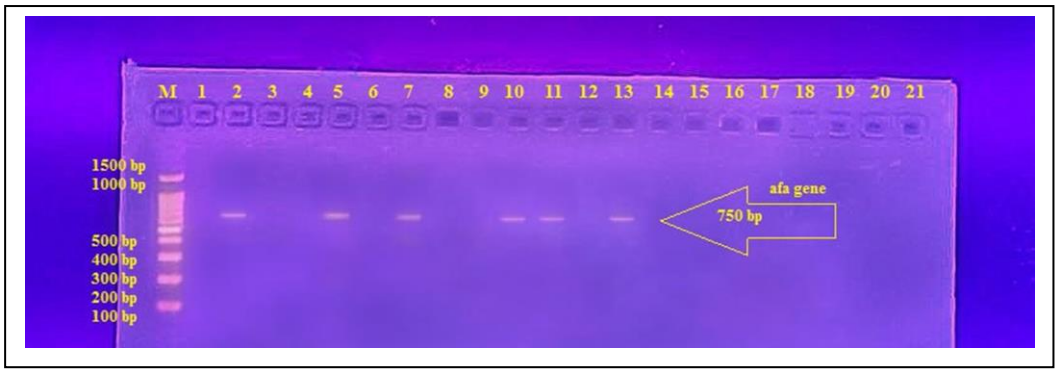

Figure 3-1 Gel electrophoresis result of *afa* gene (750 bps), were (M) indicate DNA Ladder 1500 bps.

Figure 3-2 Gel electrophoresis result of *pap* gene (328 bps), were (M) indicate DNA Ladder 1500 bps.

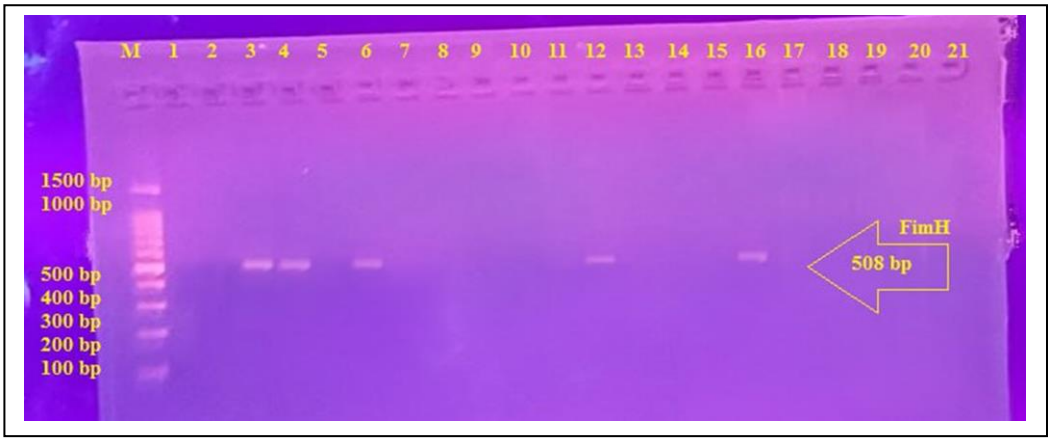

Figure 3-3 Gel electrophoresis result of *fimh* gene (508 bps), were (M) indicate DNA Ladder 1500 bps.

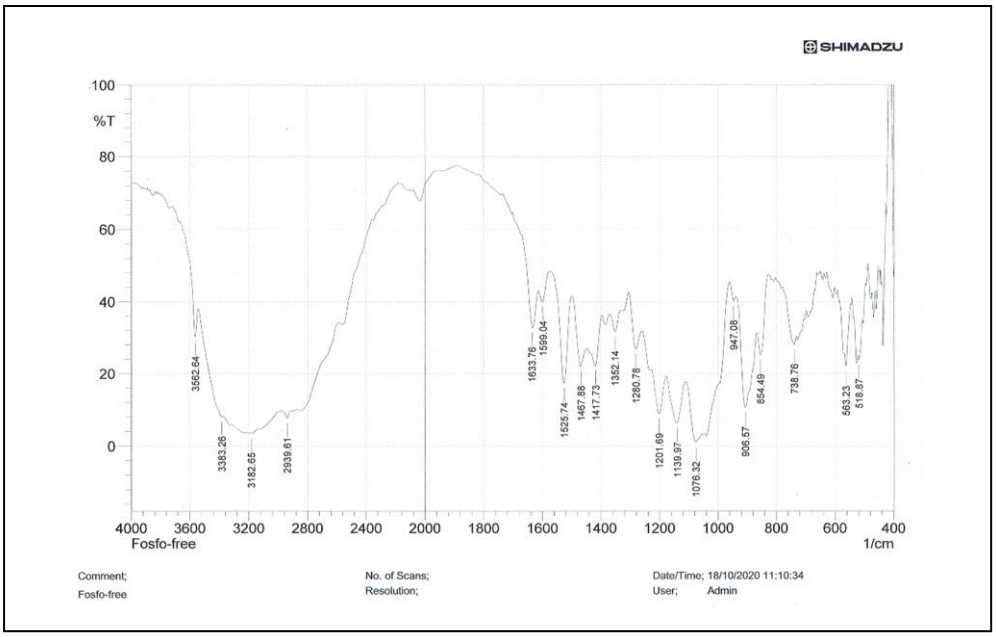

Figure 3-4: Free fosfomycin spectrum of FT-IR

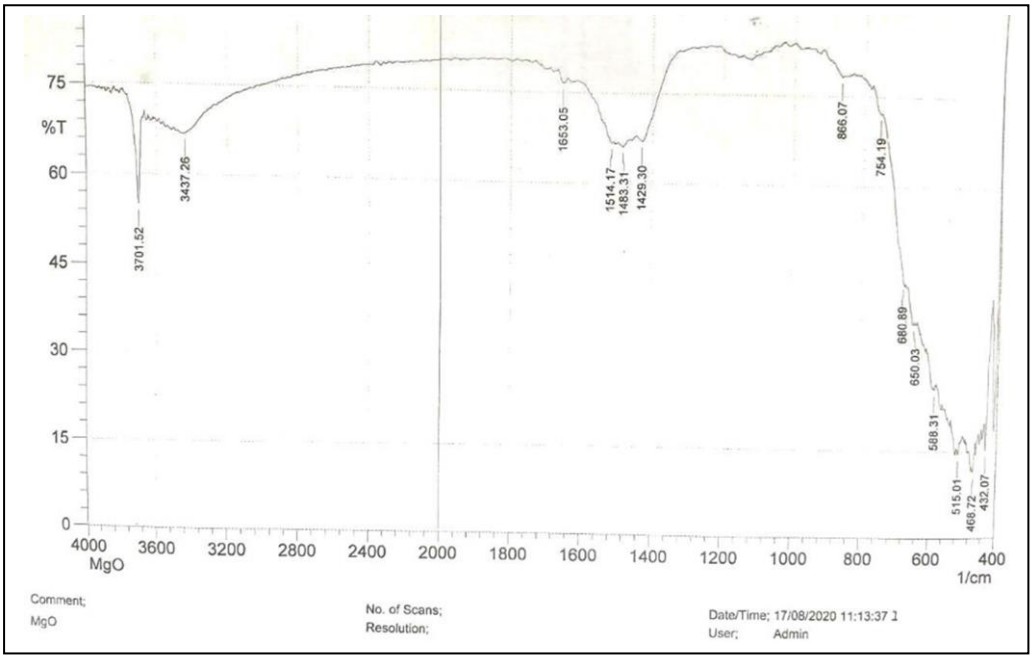

Figure 3-5 Magnesium oxide spectrum of FT-IR.

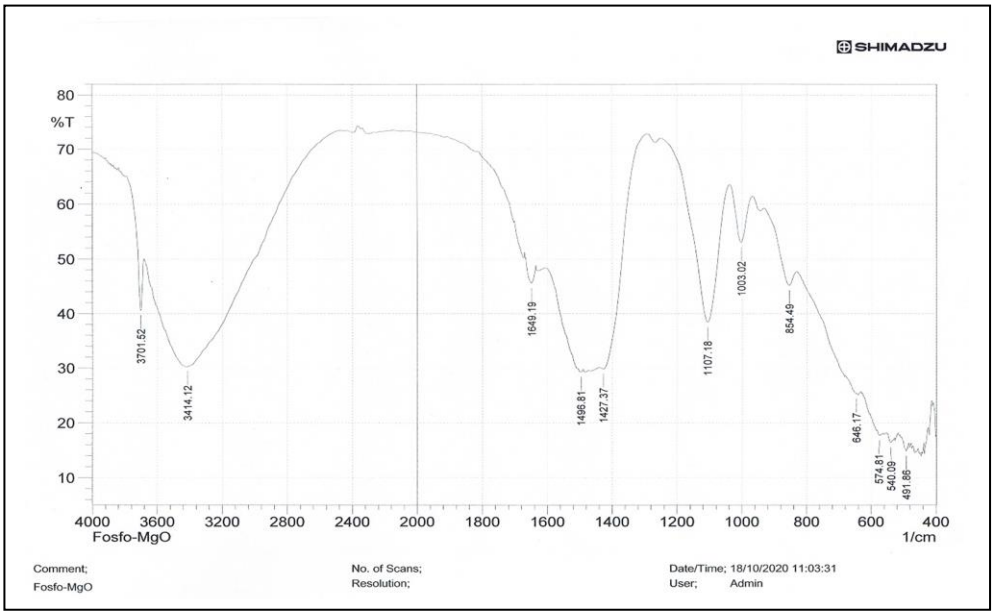

Figure 3-6 Fosfomycin-nanohybrid (Fos-MgO) spectrum of FT-IR.

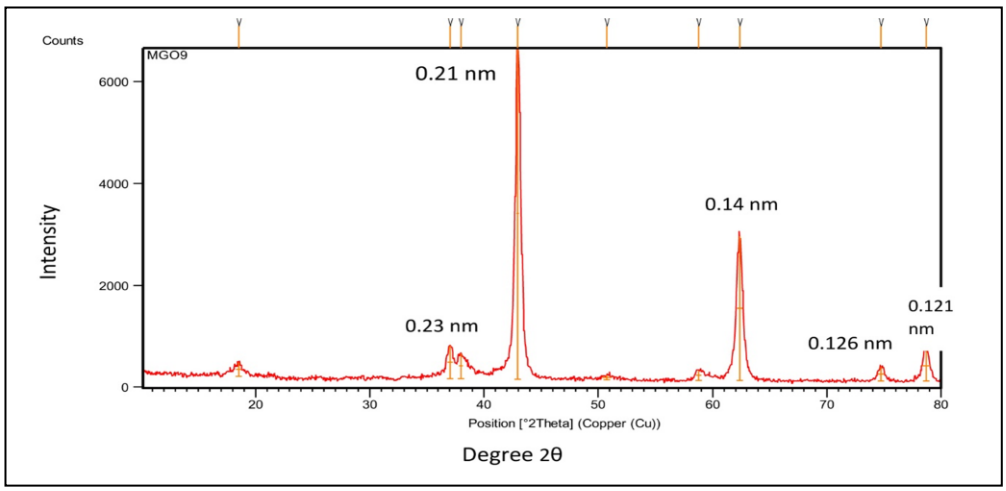

Figure 3-7 XRD spectrum of Magnesium oxide.

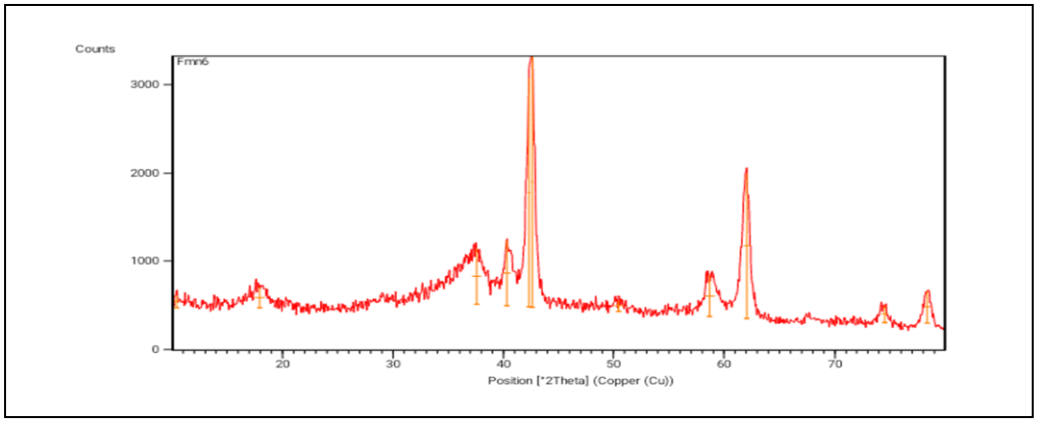

Figure 3-8 XRD spectrum of nanohybrid Fosfomycin (Fos-MgO).

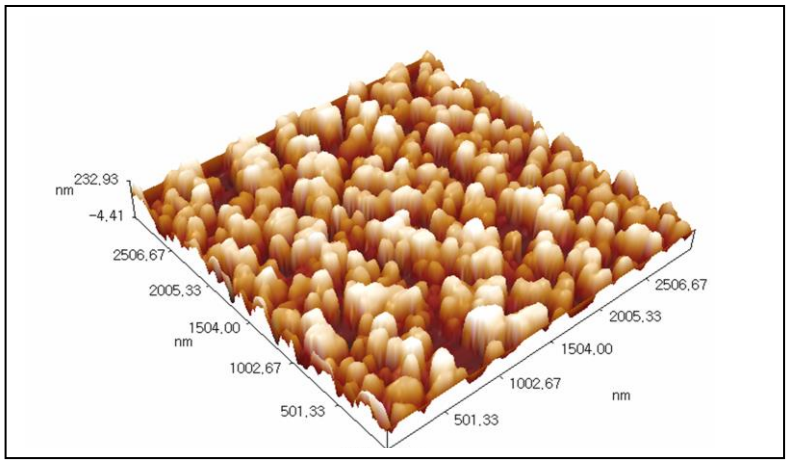

Figure 3-9 : 3D image of Fosfomycin-magnesium oxide nanohybrid (Fos-MgO) using AFM.

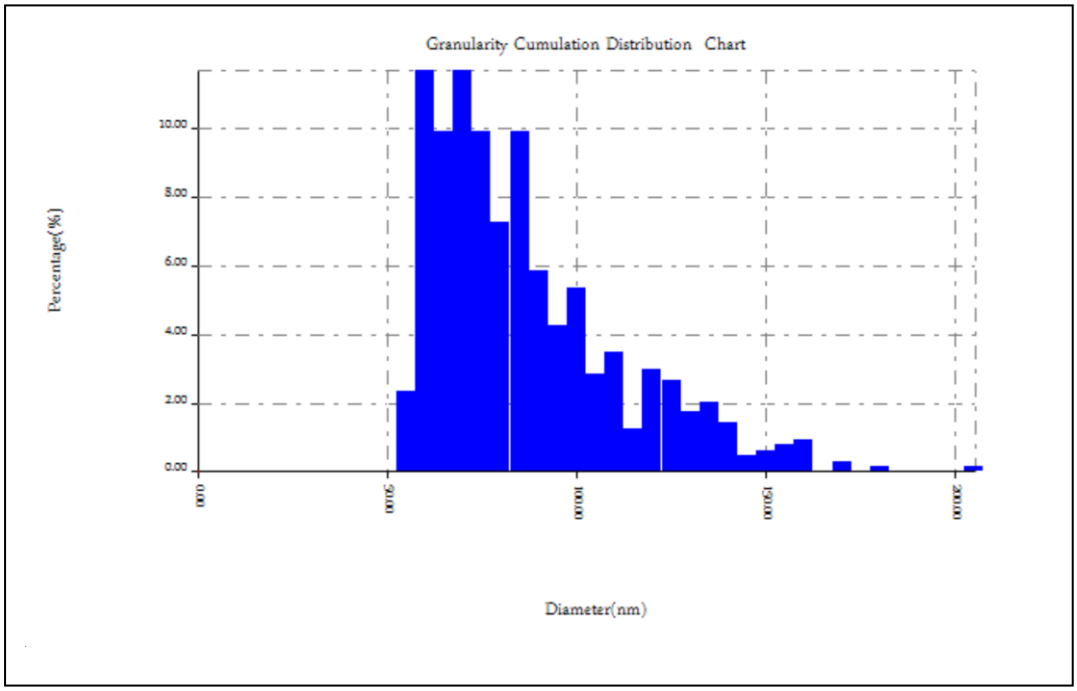

Figure 3-10 Distribution Chart of Granulity Cumulation of Fosfomycin-magnesium oxide nanohybrid (Fos-MgO) using AFM.

Table-2.1: Virulence genes primers and product size [5].			
No.	**Virulence Genes**	**Oligonucleotide sequence (5'-3') Forward and Reverse**	**Size of amplicons**
1.	***afa***	**F-GCTGGGCAGCAAACTGATAACTCTC R-CATCAAGCTGTTTGTTCGTCCGCCG**	**750 bp**
2.	***Pap***	**F-GACGGCTGTACTGCAGGGTGTGGCG R- ATATCCTTTCTGCAGGGATGCAATA**	**328 bp**
3.	***Fimh***	**F-TGCAGAACGGATAAGCCGTG R-GCAGTCACCTGCCCTCCGGTA**	**508 bp**

Table 3-1: appearance of virulence genes in specimens.

Sp. No. / Gene	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15	S16	S17	S18	S19	S20	S21	S22	S23	S24	S25
afa	-	+	-	-	+	-	+	-	-	+	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-
pap	-	+	+	-	+	-	+	-	+	-	+	-	-	+	-	-	-	-	-	-	-	-	+	+	-
fimh	-	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-

Tab.3-2: inhibition activity of free Fosfomycin

No.	Antibiotic Concentration Mg/ml / Bacterial isolate	16	32	64	128	256	512
1.	*E. coli 1*	0	11	13	17.5	23.5	26.5
2.	*E. coli 2*	9.5	12	14.5	18	21.5	24
3.	*E. coli 3*	7.5	9.5	12.5	14.5	16.5	23.5
4.	*E. coli 4*	0	9	12.5	14	19.5	23
5.	*E. coli 5*	6	10.5	14	16	19	23.5
6.	*E. coli 6*	7.5	11	14.5	16	18.5	22
7.	*E. coli 7*	10	13.5	15	16.5	21	25
8.	*E. coli 8*	11	13	17	19.5	23	25.5
9.	*E. coli 9*	8.9	12	16.5	18.8	20.5	23.5
10.	*E. coli 10*	9	12	17	19	22	24.5

Tab.3-3: inhibition activity of nanohybrid Fosfomycin (Fos-MgO)

No.	Antibiotic Concentration Mg/ml / Bacterial isolate	16	32	64	128	256	512
1.	*E. coli 1*	0	0	9	10.5	12.5	15.5
2.	*E. coli 2*	0	7.5	10.5	12.5	14	16
3.	*E. coli 3*	0	0	11	13	14	17.5
4.	*E. coli 4*	0	7	10	11	12.5	14
5.	*E. coli 5*	0	0	12	13	14.5	15
6.	*E. coli 6*	0	8	12	14	15.5	18.5
7.	*E. coli 7*	0	9.5	13	15	16.5	19.5
8.	*E. coli 8*	0	10	12.5	14	15.5	17.5
9.	*E. coli 9*	0	0	12	14.5	16.5	19
10.	*E. coli 10*	0	0	9	10.5	12.5	15

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