Molecular Detection of Some Virulence Genes and Determination the Inhibitory Efficiency of Nano-hybrid Antibiotic against *Escherichia coli*Isolated from Urinary Tract Infection.

Abstract

Objective:*Escherichia coli*is considered the most common in pathological samples related to urinary tract infections especially in women and children.It contains many virulence factors helping in pathogenicity However it is sensitive to fosfomycin that has broad spectrum in its activity against bacteria which its activity also could be confirmed using nanobiotechnology.

Methods: the study included 25 isolates of *E.coli* from patients with urinary tract infections. DNA extracted and PCR technique used for detection of 3 genes (*afa, pap* and *fimh*) coding for virulence factorafimbrial adhesions proteins, pilus rod and Type 1 fimbriae respectively.Magnesium oxide was used as a carrier in intercalating of fosfomycin between its layers.fourier transform infrared spectroscopy (FT-IR)atomic force microscope (AFM)and X-ray diffraction(XRD) were used for characterizing of prepared nanohybrid antibiotic. Antibacterial activity of against *E.coli* has been tested.

Results: PCR results showed bands with 750, 328 and 508 bps for the three genes *afa, pap* and *fimh* respectively. The all of three techniques used to confirmthe hybratingproccess indicated formation of new compound through the appearance of new group of spectrum in FT-IR, formation of spherical nanoparticles in AFMand appearance of a new diffraction planesin XRD.

Conclusion:

Keywords: Characterization;*Escherichia coli*; fosfomycin; Nanohybrid

1. Introduction

Urinary tract infections are one of the most common types of bacterial infections, with an estimated 400 million cases and 230,000 deaths globally in 2019.50% 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(Whelan *et al*., 2023).

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 (Sobel JD and Kaye D, 2014). The uropathogenicEscherichia coli (UPEC) is considered as the main cause of this type of infection, accounting for more than 75% of uncomplicated and 65% of complex infections in the urinary tract (Whelan *et al*., 2023).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(Wang *et al*., 2023).The *Afa* (afimbrialadhesin) 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 (Mishra and Panda, 2022).*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 (Whelan *et al*., 2023).

Fosfomycin is one of the most important broad-spectrum bactericidal antibiotic used for treatment of UTI and multidrug-resistant bacterial infection (Falagas*et al*., 2016).

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 (Tang and Lv, 2014).

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. 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 (Jabur, 2021).

The current study aimed to prepare an efficient nanohybrid antibiotic using fosfomycin inhibiting virulence UTI isolate.

2. Material and methods

2.1. 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 was used for extraction of DNA from the isolates according to the manufacturer instruction (http://addbio.net/default/subc/c03.php?com_board_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 (2022) study. DNA purity was checked by Nano-drop. Virulence genes under study are shown in Table (1).

Table-1:-Virulence genes primers and product size(Mishra and Panda, 2022).

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**

The amplification conditions for the virulence genes illustrated in Table2 .

Table-.2:- Amplification conditions of the PCR-based analysis for the detection of different uropathogenic markers.

Cycle	Step		Temperature (°C)	Time
1	Initial denaturation		95	5 min
35	Denaturation		95	35Sec
	Annealing for	*afa*	55	1 min
		pap	57	
		Fimh	60	
	Extension		72	60 Sec
1	Final extension		72	7 min

Preparation of nanohybrid Fosfomycin:

The nanohybrid Fosfomycin was prepared according to the method that described by Bashi *et al*. (2013) as follows:

a- Preparation of Fosfomycin solution

This solution was prepared by dissolving 1.2 gm of Fosfomycin in 40 ml of suitable solvent and the volume completed to 50 ml with same solvent, too.

b- Preparation of carrier:

Carrier solution was prepared by dissolving 1 gm of magnesium oxide (MgO) in 50 ml of 50% ethanol.

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 nanohybrid Fosfomycin:

The nanohybrid Fosfomycin of present study was characterized by using several methods according to (Silverstein *et al*., 2005; Mir *et al*., 2012) as follows:

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

The infrared spectrum study for each of nanohybrid Fosfomycin and Fosfomycin free form as well as MgO was carried out by making disks through mixing with potassium bromide (KBr) after grinding well. The infrared spectrum was measured in a wave number range (4000-400) cm^{-1}.

(ii) X-ray diffraction spectrum (XRD):

The nanohybrid Fosfomycin of the present study was characterized using XRD which explains the difference in the layer before and after the intercalating process by using Bragg's law.

(iii) Atomic force microscope (AFM)

The current study included measuring the diameter, size and aggregation of the nanohybrid Fosfomycin nanoparticles by using AFM.

Detection of inhibitory activity of free and nanohybrid Fosfomycin:

The inhibitory activity of free and nanohybrid form of Fosfomycin was detected against *Escherichia coli*isolated from urinary tract infections according to the well diffusion method (Egorov, 1985) by using antibiotic concentration of (16-512) µg/ml.

3. Results and Discussion

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

The results in following images show 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 3show appearance of virulence genes in specimens.

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

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

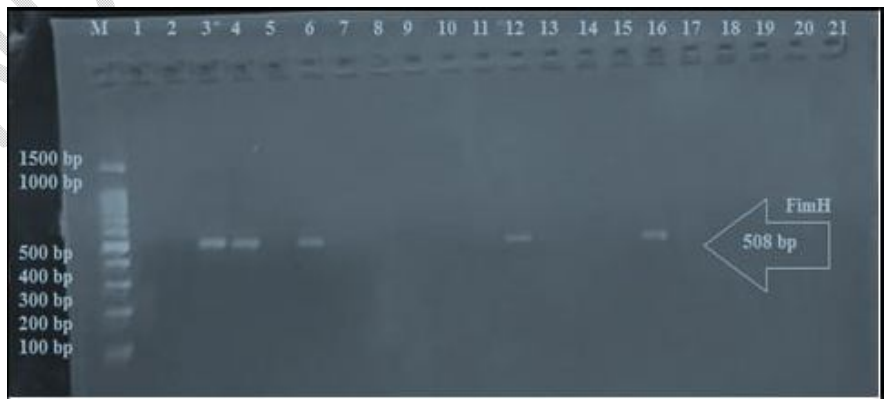

Figure -3 Gel electrophoresis result of *fimh* gene (508 bps), were (M) indicate DNA Ladder 1500 bps.

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).

Table 3: 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	-	-	+	+	-	+	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-	-	-	+	-

Many virulence factors have a role in the pathogenicity of *Esherishia coli* in urinary tract infection of which adhesion proteins have a prime function. Type 1 fimbriae are encoding result of the *fim*operon cluster and consist of a prime protein, FimA, associated with supplementary proteins FimGand FimFand the adhesion protein Fimh.*pap* gene cluster is an aggregation of 11 genes that encode for main element of the pilus rodThe five defined *afaA* to *E* 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. (Rahdar et al., 2015).

Characterization of nanohybrid antibiotics

1. Characterization by FT-IR:

Figure 4 shows the results of FT-IR spectrum of free Fosfomycin. The broad band that appeared at (3182) cm^{-1} was attributed to a stretch vibration of the two hydroxyl groups. The appearance of the band at frequency of (2939) cm^{-1} was due to the aliphatic (C-H) stretch.The band which appeared at 1633 cm^{-1}includes the stretch of P=O group. In addition, the appearance of the two bands at (1139 and 1076) cm^{-1} indicate the stretch of etheric C - O – C in the epoxide ring (Silverstein *et al.*, 2005).

Figure 5 showsfew bands of absorption in the FT-IR spectrum of magnesium oxide (MgO). The band at 3437 cm^{-1} is related toO-H group's stretching vibration whichassociated to the physically adsorbed water, while the band at 1514 cm^{-1}related to the bending vibration of O-H group surface of the mentioned absorbed water. Anyhow the band at 1429 cm^{-1} is related to the asymmetric stretching of (CO_3^{-2}) andthe vibration of metal bond was showed at (680-580) cm^{-1} (Fardood*et al*. 2018).

Figure 6 shows a combination FT-IR spectrum of both the Fosfomycin and MgO carrier. 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 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} (Silverstein *et al*., 2005).

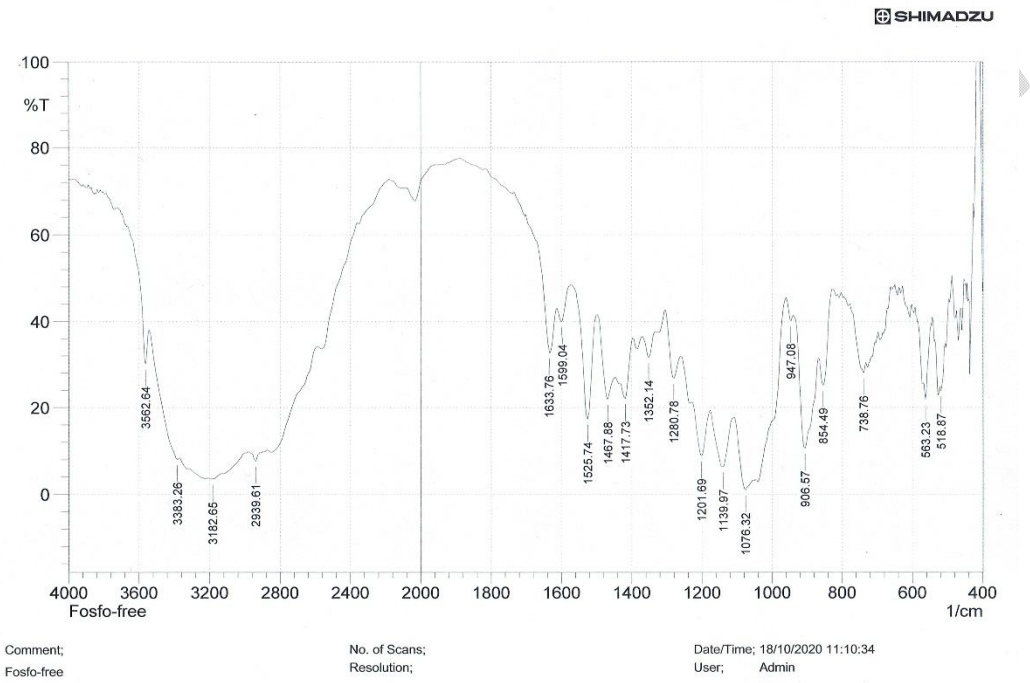

Figure-4 FT-IR spectrum of free Fosfomycin.

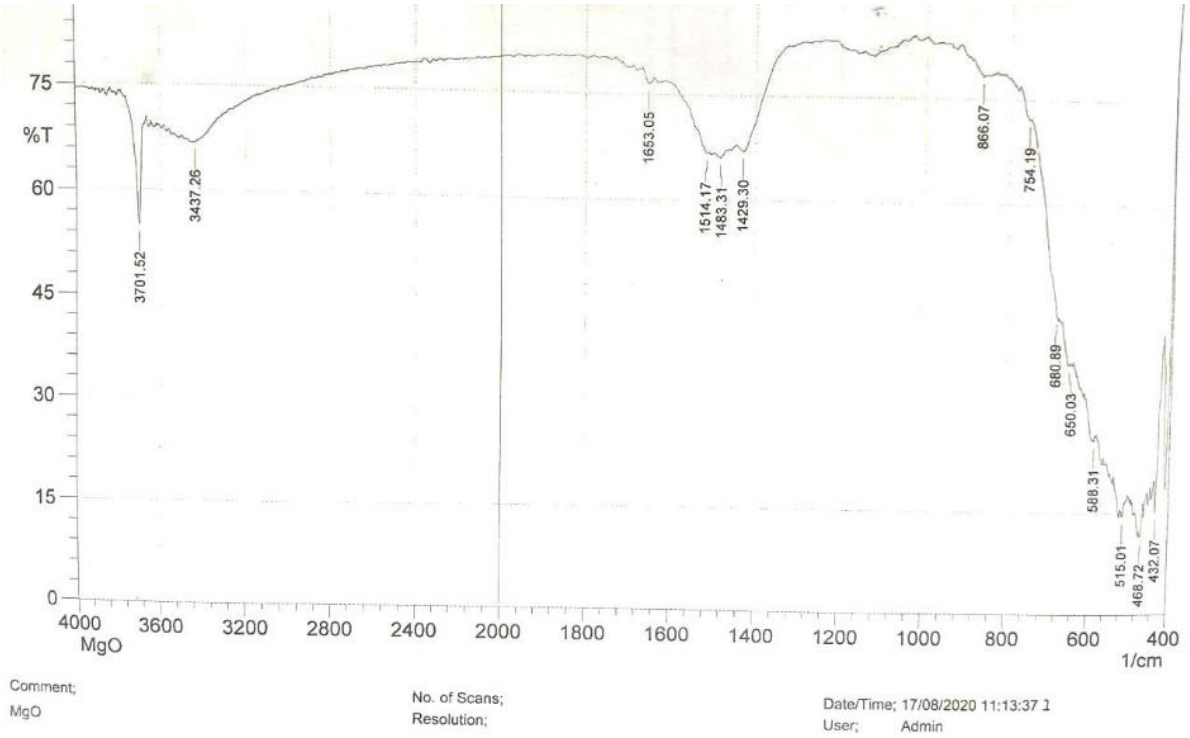

Figure-5 FT-IR spectrum of Magnesium oxide.

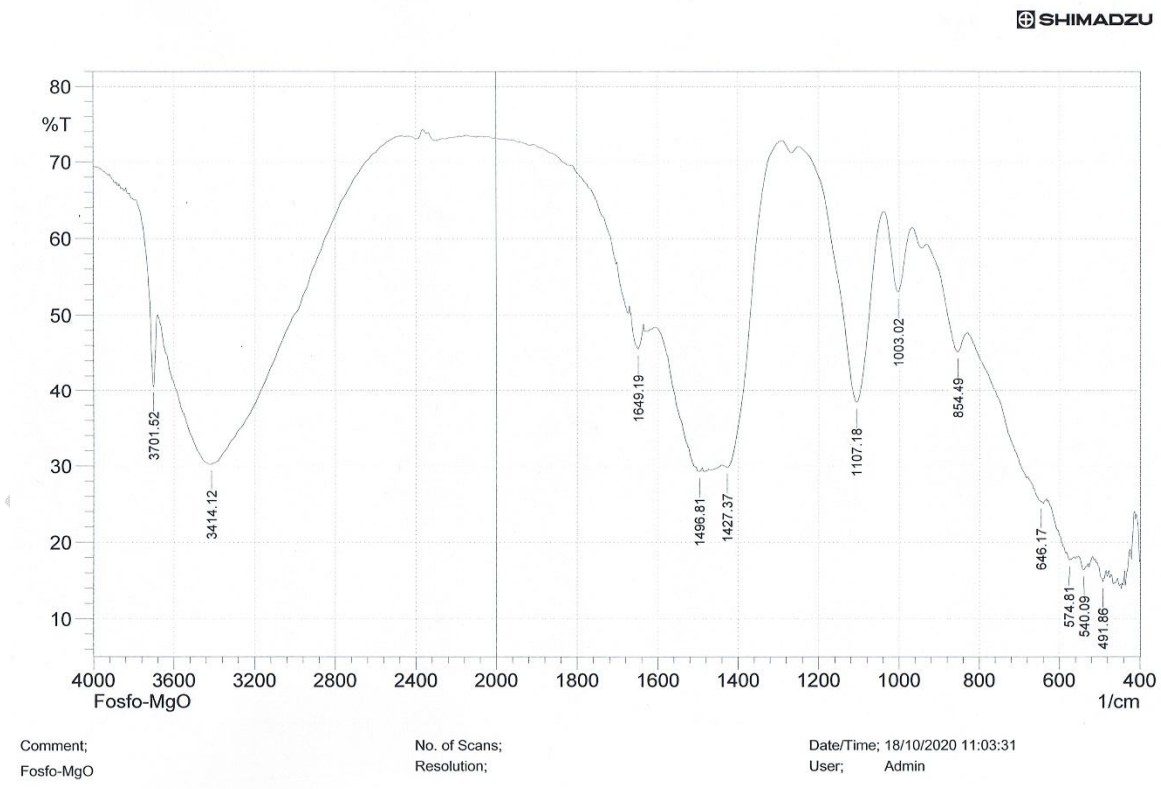

Figure6 FT-IR spectrum of nanohybrid Fosfomycin (Fos-MgO).

2. Characterization by XRD:

In current study diffraction spectrum of X-ray of MgO alone as well as Fos-Mgo were measured to obtain the differences in the layer thickness before and after loading of fosfomycinon MgO layers by using Bragg's law.

Figure7 shows XRD analysis of MgO. As shown, many diffraction planes are appeared in the spectrum,as follows:At37.95°, (111) with crystalline distance of 0.23 nm, at 42.95°, (200) with crystalline distance of 0.21 nm, at 62.37°, (220) with crystalline distance of 0.14 nm, at 74.75°, (311) with crystalline distance of 0.126 nm and at 78.66° the diffraction plane was (222) with crystalline distance of 0.121 nm (Fardood*et al*,. 2018).

Figure 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 appeared 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 magnesium oxide to compose Fos-MgO.

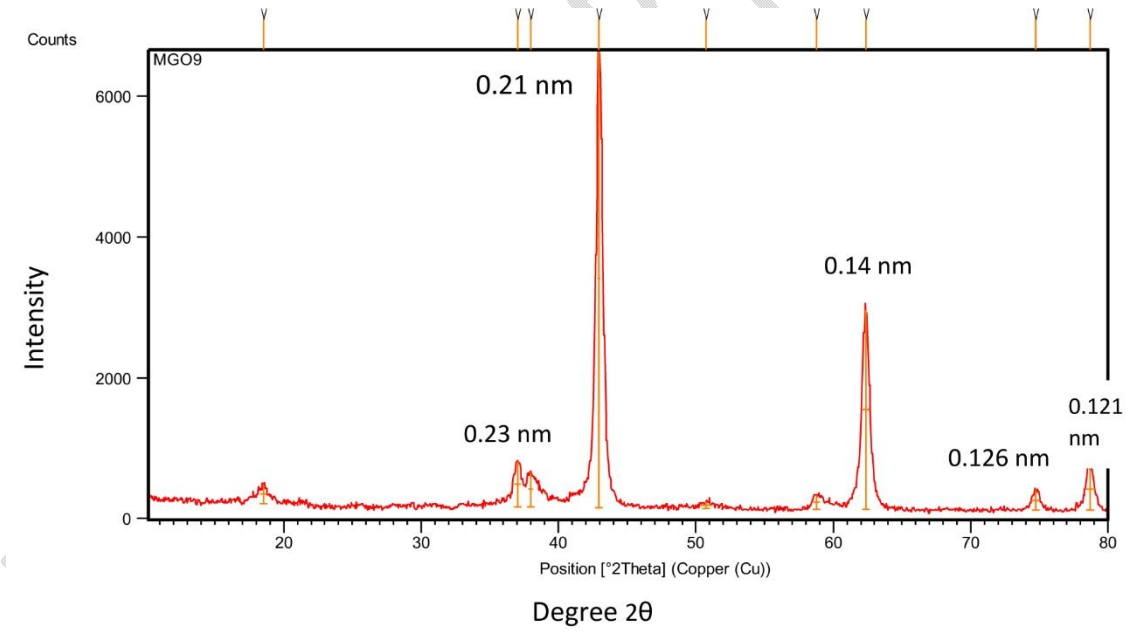

Figure7 XRD spectrum of Magnesium oxide.

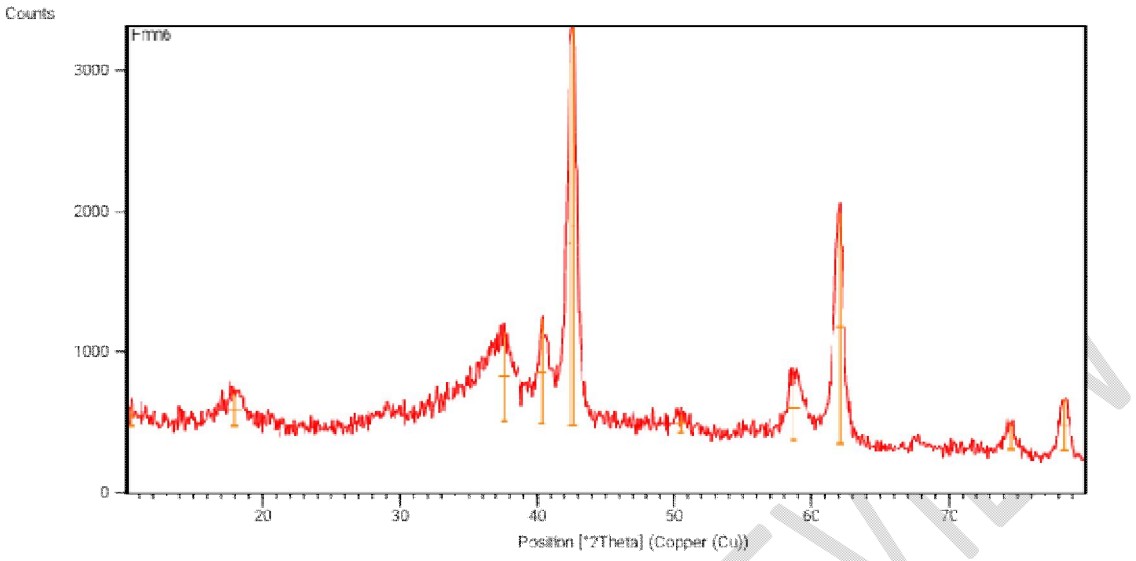

Figure-8 XRD spectrum of nanohybrid Fosfomycin (Fos-MgO).

3. Characterization by AFM

The outer surface of Fos-MgO was scanned by Atomic force microscope (AFM).

Figure9 show the three dimensional section of prepared nanoantibiotic. The nanoparticles were almost spherical in shape with height of 232 nm. Figure 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 (2021)who obtained a diameter average size of 77 nm for the prepared nanohybrid ciprofloxacin loaded on MgO.

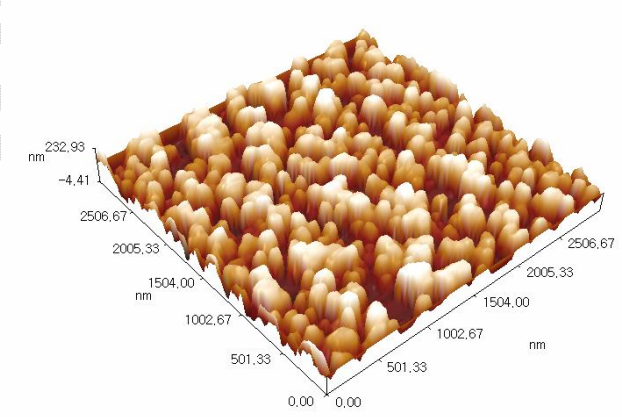

Figure-9 Three Dimensional image of nanohybrid Fosfomycin (Fos-MgO) by Atomic Force Microscope.

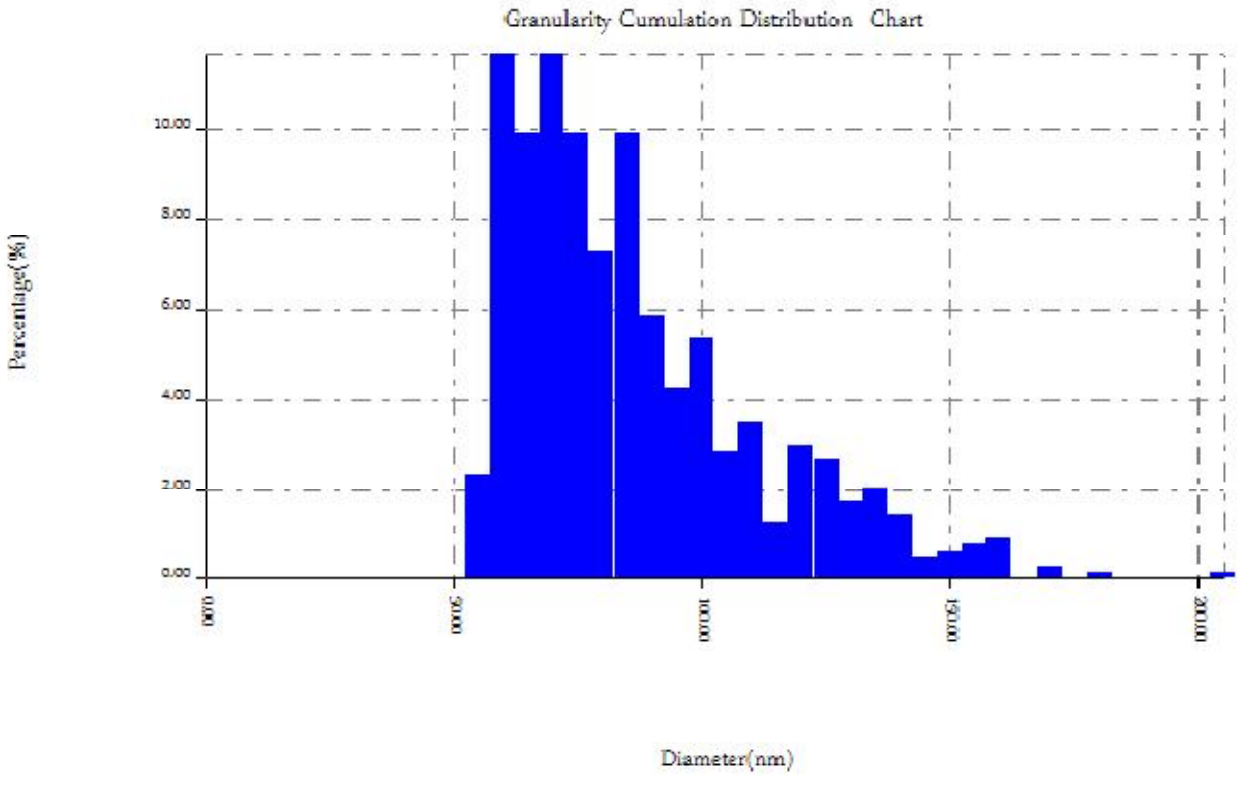

Figure 10 Granulity Cumulation Distribution Chart of nanohybrid Fosfomycin (Fos-MgO) by Atomic Force Microscope.

4. The inhibition activity of the nanohybrid antibiotic against *E. coli*:

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) (Falagas et al., 2016).The inhibition activity of free and the nanohybrid antibiotic (Fos-MgO) was studied against ten isolates of *E. coli*isolated urinary tract infections. Table 4 shows the inhibition activity of free fosfomycin. The diameters size indicate 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 4 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 least inhibition diameter of 22 mm on *E. coli*6 isolate.

by comparing the diameter of the inhibition zone for both the free and hybrid antibiotics with magnesium oxide on the same isolate in Tables 4 and 5, it is noted that the diameter of inhibition for the free antibiotic is larger than the nano-hybrid antibiotic for the same concentration, but it must also be noted that in the case of nano-hybrid the percentage of antibiotic loaded on the carrier is approximately one-third of the percentage of free antibiotic used for inhibition.

Many studies applied to comparing between the inhibitory activity of free antibiotics and that of nanohybrid one against pathogenic bacteria. In study conducted by Al-Fatlawi (2021) it has been shown that the nanohybrid antibiotic was very efficient in inhibiting of isolates. However she studied the effect of azithromycin of *Klebsiella pneumonia* isolates. also our results was similar to those announced by Kumar *et al.* (2016) where found the high efficiency of doxycycline nanoparticle in the inhibiting of *E. coli*isolates compared to that of free doxycycline.

Tab.3-2: inhibition activity of free Fosfomycin

No.	Antibiotic Concentration Mg/ml / Bacterial isolate	16	32	64	128	256	512
1.	*Escherichia coli 1*	0	11	13	17.5	23.5	26.5
2.	*Escherichia coli 2*	9.5	12	14.5	18	21.5	24
3.	*Escherichia coli 3*	7.5	9.5	12.5	14.5	16.5	23.5
4.	*Escherichia coli 4*	0	9	12.5	14	19.5	23
5.	*Escherichia coli 5*	6	10.5	14	16	19	23.5
6.	*Escherichia coli 6*	7.5	11	14.5	16	18.5	22
7.	*Escherichia coli 7*	10	13.5	15	16.5	21	25
8.	*Escherichia coli 8*	11	13	17	19.5	23	25.5
9.	*Escherichia coli 9*	8.9	12	16.5	18.8	20.5	23.5
10.	*Escherichia 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
11.	*Escherichia coli 1*	0	0	9	10.5	12.5	15.5
12.	*Escherichia coli 2*	0	7.5	10.5	12.5	14	16
13.	*Escherichia coli 3*	0	0	11	13	14	17.5
14.	*Escherichia coli 4*	0	7	10	11	12.5	14
15.	*Escherichia coli 5*	0	0	12	13	14.5	15

16.	***Escherichia coli 6***	**0**	**8**	**12**	**14**	**15.5**	**18.5**
17.	***Escherichia coli 7***	**0**	**9.5**	**13**	**15**	**16.5**	**19.5**
18.	***Escherichia coli 8***	**0**	**10**	**12.5**	**14**	**15.5**	**17.5**
19.	***Escherichia coli 9***	**0**	**0**	**12**	**14.5**	**16.5**	**19**
20.	***Escherichia coli 10***	**0**	**0**	**9**	**10.5**	**12.5**	**15**

4. References

Al-Fatlawi NG, AL-Ghanimi AA. Preparation and characterization of nano hybrid azithromycin and evaluate its inhibitory activity on locally isolated *Klebsiella pneumonia*.Biochem Cell Arch. 2021;21(2):4775-4783

Bashi AM, Hussein MZ, Zainal Z, Tichit D. Synthesis and controlled release properties of 2, 4-dichlorophenoxy acetate–zinc layered hydroxide nanohybrid. Journal of Solid State Chemistry. 2013 Jul 1;203:19-24.

Egorov NS. Antibiotics: A scientific approach. Mir publishers; 1985.

Falagas, M.E., Vouloumanou, E.K., Samonis, G. and Vardakas, K.Z., 2016. Fosfomycin. *Clinical microbiology reviews*, *29*(2), pp.321-347.

Fardood S, Ramazani A, Woo Joo S. Eco-friendly synthesis of magnesium oxide nanoparticles using arabic Gum. Journal of Applied Chemical Research. 2018 Jan 1;12(1):8-15.

Jabur, N. G. (2021). Isolation and identification of some bacterial species causing respiratory tract infections and evaluation their inhibitory efficiency using nanoprepared azithromycin and ciprofloxacin. M.Sc. thesis College of Science, University of Kerbala, Kerbala/ Iraq.

Kumar NI, Das SA, Jyoti AN, Kaushik SA. Synergistic effect of silver nanoparticles with doxycycline against *Klebsiella pneumoniae*. Int J Pharm Pharm Sci. 2016;8(7):183-6.

Mir M, Leite FL, Herrmann Junior PS, Pissetti FL, Rossi AM, Moreira EL, Mascarenhas YP. XRD, AFM, IR and TGA study of nanostructured hydroxyapatite. Materials Research. 2012 Aug;15(4):622-7.

Mishra, S. and Panda, G., 2022. Molecular Characterization of Uropathogenic Markers in Escherichia coli Isolated from Pregnant Women having Urinary Tract Infection. *International Journal of Creative Research Thoughts*, *10*(2), pp.540-554.

Rahdar, M., Rashki, A., Miri, H.R. and Ghalehnoo, M.R., 2015. Detection of pap, sfa, afa, foc, and fim adhesin-encoding operons in uropathogenic Escherichia coli isolates collected from patients with urinary tract infection. *Jundishapur journal of microbiology*, *8*(8).

Silverstein RM, Webstar FX,Kiemlie DJ. Spectrometric Identification of Organic Compounds [Internet]. 7 Ed. John Wily and Sons. INC. Printed in United States of America. 2005.

Sobel, J.D., Kaye D. (2014) *Principles and Practice of Infectious Diseases*, 886-913, Elsevier Saunders, Philadelphia.

Tang, Z.X. and Lv, B.F., 2014. MgO nanoparticles as antibacterial agent: preparation and activity. *Brazilian Journal of Chemical Engineering*, *31*, pp.591-601.

Wang, M.C., Fan, Y.H., Zhang, Y.Z., Bregente, C.J.B., Lin, W.H., Chen, C.A., Lin, T.P. and Kao, C.Y., 2023. Characterization of uropathogenic Escherichia coli phylogroups associated with antimicrobial resistance, virulence factor distribution, and virulence-related phenotypes. *Infection, Genetics and Evolution*, *114*, p.105493.

Whelan, S., Lucey, B. and Finn, K., 2023. Uropathogenic Escherichia coli (UPEC)-Associated Urinary Tract Infections: The Molecular Basis for Challenges to Effective Treatment. *Microorganisms*, *11*(9), p.2169.