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Synthesis and Characterization of Florfenicol-Silver Nanocomposite and its Antibacterial Activity against some Gram Positive and Gram-Negative Bacteria

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ABSTRACT

In this paper, easy, rapid and cheap synthetic method was described for florfenicol-silver nanocomposite by sonochemical method. Florfenicol-silver nanocomposite was characterized based on three classes namely index, identification and morphology class. Index characterization was carried out by zeta sizing, BET surface area and zeta potential. Identification characterization was performed using X-ray diffraction (XRD) and Raman spectrometry. Morphology characterization was done utilizing transmission electron microscope (TEM), scanning electron microscope (SEM) and atomic force microscope (AFM). Characterization results showed zeta sizing of florfenicol was 30.44nm, while florfenicol-silver nanocomposite was 33.5 nm with zeta potential -14.1 and -18, respectively. BET surface area was found to be 13.3, 73.2 and 103.69 m^2/g for florfenicol, silver nanoparticles and florfenicol-silver nanocomposite respectively. XRD and Raman charts confirmed the formation of florfenicol-silver nanocomposite without any contamination. TEM, SEM and AFM spectral data illustrated spherical to sub spherical shape of silver nanoparticles on cubic to sheet shape of florfenicol with size less than 50 nm. Antimicrobial activity was screened where the average zone of inhibitions caused by the prepared nanocomposite were 28.3 mm, 24 mm, 27.3 mm and 24 mm compared to 17.7 mm, 16 mm, 18.7 mm and 13.3 mm of the native drug and 13 mm, 10 mm, 14.3 mm and 15 mm of the used positive reference standards against *E. coli*, *Salmonella typhymurium*, *Staphylococcus aureus* and *Staph.aureus* MRSA respectively.

Key words: Florfenicol-silver nanocomposite; Characterization; Antimicrobial activity.

INTRODUCTION

Around 1970 nanotechnology was initially created for assembling novel materials their size ranged from 1 to 1000 nanometer (Lang, 2006; AbdElgadir *et al*., 2015; Youssef *et al*., 2019). In addition, it participates in many fields such as; agriculture, biology and drug delivery (Scott *et al*., 2007; Zhang *et al*., 2008; Underwood and Van 2012; Youssef *et al.*, 2019). Nanoparticles are viewed as gainful than the mass materials because of their enormous surface to volume proportion, higher reactivity, solvency, bioactivity, controlled molecule size, controlled arrival of medications, site-explicit focusing on and bioavailability (Peer *et al*., 2003., Berger *et al*., 2004; Mohanraj and Chen, 2006, Catarina *et al*., 2006., Moudgil and Ying 2007, Buzea *et al*., 2007, Hahens *et al*., 2007, Jin and Hu. 2008; Bentolila *et al*., 2009, Zadeh and Moradi Kor 2013, Num & Useh, 2013 and Rassouli *et al*., 2016; Youssef *et al*., 2019).

Silver nanoparticles were synthesized by different methods such as precipitation, sonochemical and solvohermal methods (Sajjad and Nasseri, 2011, Brajesh *et al*., 2014 and Rosemary and Pradeep, 2003). Some nanoparticles such as silver nanoparticles at low concentrations were nontoxic to humans, it has been reported that silver nanoparticles were effective on some bacteria like; *E. coli*, *S. aureus*, *Klebsiella* and *Pseudomonas* by attacking their respiratory chain and cell division resulting in cell death (Kim *et al*., 2013; Sharma *et al*., 2018; Youssef *et al.*, 2019). In addition, silver nanoparticles accelerated the healing of *S. aureus* infected skin wounds in mice without side effects in mice (Adibhesami *et al.*, 2017). Florfenicol is used in veterinary medicine as a broad-spectrum antibiotic which act against gram-positive and gram-negative bacterial infections. It is widely used for prevention and treatment of the respiratory diseases in cattle, vibriosis in fish, swine and chicken diseases.

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In addition, it is used for treatment of (Typhoid fever, paratyphoid, Haemophilus influenzae, pneumonia caused by β- lactamase producing strains, Meningeococcal infections, Anaerobic or mixed infections as in sever rickettsial infection (Youssef *et al.,* 2019).

Its mechanism of action is similar to thiamphenicol and chloramphenicol. The use of florfenicol not result in aplastic anaemia (Shin *et al.,* 2005; Jiang *et al.,* 2006; Anadon *et al.,* 2008; Youssef *et al.,* 2019). There are some disadvantages of florfenicol, firstly its poor solubility in aqueous solutions so organic solvents are usually used in its clinical aqueous formulations. Although organic solvents are able to increase the solubility of the drug, they increase adverse effects such as irritation and toxicity. In addition, in some animals the elimination half-time of florfenicol is less than 3 hours and frequent dosage is needed in clinical applications to obtain better therapeutic efficacies. Frequent administration would increase labour cost and animal stress. Therefore, development of florfenicol novel formulations will have promising practical values (Ali *et al.,* 2003; Shen *et al.,* 2003; Tao *et al.,* 2014; Anadon *et al.,* 2008; Youssef *et al.,* 2019).

There are a few hindrances of florfenicol, initially its poor solubility in water so natural solvents are normally utilized in its clinical watery definitions. Albeit natural solvents can expand the dissolvability of the medication but they increase the toxicity. What's more, in certain creatures the disposal half-time of florfenicol is under three hours and incessant dose is required in clinical applications to get better helpful efficacies. Along these lines, advancement of florfenicol novel preparations will have promising handy qualities (Youssef *et al.,* 2019).

Song *et al.,* (2010) reported that florfenicol loaded on silica nanoparticles showed a slower sustained release than the rapid release of native florfenicol. Wang *et al.,* (2015) Prepared florfenicol-loaded solid lipid nanoparticle by hot homogenization and ultrasonic technique. Results showed that zeta potential approximately was more than 47 mV. Minimum inhibitory concentration (MIC) was 3 μg per mL against *E. coli*.

This work aims to synthesis the florfenicol-silver nanocomposite and characterize the formed nanocomposite using zeta potential and nanosizer, SEM, TEM, AFM and BET surface area. In addition, investigation of the antibacterial activity of the prepared nanocomposite against both gram-positive and gram-negative bacteria in comparison with the native drug.

MATERIALS AND METHODS

Materials

All chemicals are lab grade and used without any purification. Silver nitrate and trisodium citrate were manufactured by Sigma Company, India. Florfenicol powder was supplied by Pharma Sweed Company.

Methods

Synthesis of silver nanoparticles

Silver nitrate and trisodium citrate were used as precursor materials forthe synthesis of silver nanoparticles. The silver colloid was synthesized by using co-precipitation method. In typical experiment, 50 ml of 0.001 M $AgNO_3$ was heated to boil then 5 mL of 1% trisodium citrate solution was added drop by drop. During the process, solutions were mixed vigorously and heated until change of color was obtained (pale yellow). Then it was removed from the heating device and stirred until cooled to room temperature and collected in dark bottles to avoid the effect of lights (Sajjad and Nasseri, 2011). The mechanism of reaction could be expressed as follows:

$$4Ag^+ + C_6H_5O_7Na_3 + 2H_2O \rightarrow 4AgO + C_6H_5O_7H_3 + 3Na^+ + H^+ + O_2\uparrow$$

Synthesis of florfenicol-silver nanocomposite

0.2 g of florfenicol powder was added to 100 ml doubled distilled water, stirred at 1000 rpm at 40 °C and stirred for 20 minutes; then, 5 ml Ag nanoparticles (100 ppm) was added and mixed well and entered in ultra-sonic apparatus for nanocomposite formation under the following conditions: plus every 3 seconds at 80% amplitude power maximum temperature for 60 minutes.

Characterization

The aim of characterization of florfenicol-silver nanocomposite was not only determine physico-chemical properties but also influence characters to its bioactivity. Characterization was classified into three classes namely index, identification and morphology classes. Composition class was done by XRD (D8 Discovery – Bruker Company) to confirm presence of crystal structure, crystal size and give an information about crystal phase using thin film which created by spin coating instrument, condition of measurement was 40 KV and 40 AM (1600W) at speed scan 0.02 and 2theta range from 5◦ to 80◦. Raman spectra were performed using Lab. RAM-HR Evolution Horiba Co., of air-cooled open electrode 1024 x 256-pixel CCD detector and equipped visible single spectrometer. The 532 nm He–Cd edge laser line with grating 1800 (450-850 nm) and ND filter 3.2% was used to avoid burning of sample with acquisition time 10 sec, accumulations 5 without delay time and spike filter and objective was X100_VIS. Microscopic class was carried out by using transmission electron microscope (TEM) (model EM-2100 High-Resolution-Japan) at magnification 20X and voltage 200 kV and was carried out to confirm 2D shape and size. Also, scanning electron microscope (SEM) was carried out using Jol 2000, Japan, and it was carried out to confirm 2D shape. AFM (5600LS Agilent Technology Company) was used to confirm 2D and 3D roughness profile and particles. Preparation of sample before measurement on AFM was done by adding sample in the form of powder in water and sonication for 45 minutes. The suspension was added on mica slide, at condition of measurement (size 500 X 500 nm, speed 0.5 inch/sec, I Gain = 0.5 and P Gain = 20 using contact mode). Index class was carried out to determine the BET surface area (degassing was done to remove moisture content and dust at 100 °C under vacuum (10 °C /min for 1 hour then soaked for 1 hour) using surface area and pore size analyzer model Nova Touch LX2 manufactured by Quantachrome Company. Zeta potential (to confirm charge of the sample to know if it is colloidal or not) and sizer was also determined in this sector by Nano Sight NS500 manufactured by Malvern, UK.

Antibacterial activity assay

Florfenicol-silver nanocomposite was tested for its antimicrobial activity against Gram positive and Gram-negative bacteria in comparison with its native form (florfenicol) by the disc diffusion method. According to the standard protocol described by the [National Committee for Clinical Laboratory Standards] Bacterial suspension was prepared as follows: a colony was grown in nutrient broth overnight. Turbidity was matched with McFarland Standard 0.5. Then, the colony was cultured on Mueller Hinton agar medium Table 1 (Balouiri *et al*., 2016).

Paper discs (6 mm in diameter) were impregnated with 30 µL of the tested preparation which concentration was 1mg/ml and placed on Mueller Hinton agar plates, which were inoculated with test organisms [National Committee for Clinical Laboratory Standards]. The plates were incubated at 37°C for 24 hours and the diameters of the inhibition zones were measured. Each assay was performed in triplicate and repeated three times.

Table 1: The culture medium, incubation condition and reference standard used for each micro-organism.

Microbial type	Medium	Incubation condition
Escherichia coli (ATCC35218)	Mueller	37°C for
Salmonella typhymurium (ATCC14028)	Hinton	24 hours
Staph. aureus (ATCC29213)	Agar	
Staph.aureus MRSA (ATCC43300)		

RESULTS AND DISCUSSION

Identification class

XRD

XRD was carried out not only for florfenicol-silver nanocomposite but also to silver and florfenicol to confirm the formation of florfenicol-silver nanocomposite without any shifting of its 2theta peaks position or presences of peaks of other chemicals used through the synthesis process. The data obtained indicated the purity of florfenicol-silver nanocomposite as illustrated in Figure (1) which pointed out the presence of 29 characteristic peaks for florfenicol, 11 peaks for florfenicol-silver nanocomposite and zero characteristic peaks for silver nanoparticles as shown in table 2. The XRD results gave information about crystalline and crystal form of materials. The XRD curve of AGNPs showed best amorphous state (zero peaks) without any addition peaks indicated the homogeny of chemical composition. The decrease in the number of characteristic peaks for florfenicol-silver nanocomposite than florfenicol was due to effect of amorphous silver nanoparticles on intensity of florfenicol crystals.

Raman spectra

To confirm the formation of florfenicol-silver nanocomposite, Raman shift measurement must be carried out to silver nanoparticles, florfenicol and florfenicol-silver nanocomposite as shown in Figure (2). Raman spectrum of florfenicol illustrated 18 characteristic peaks at 36.9, 53.4, 78.6, 146.9, 225.7, 257.4, 325.8, 360.1, 473.97, 630.1, 720.4, 769.1, 816.1, 856.1, 879.95, 989.3, 1101.57, 1139.74, 1196.11, 1302.4, 1438.34, 1598.24, 1680.4, 2920.85 and 2987.04 cm^{-1}, respectively. The 36.9,

Table 2: 2theta angle and d-space values of florfenicol and florfenicol-silver nanocomposite.

Index	Angle	d value	Angle	d value
	Florfenicol		Florfenicol-Silver nanocomposite	
1	8.040 °	10.98773 Å	7.988 °	11.05911 Å
2	16.171 °	5.47680 Å	8.046 °	10.97958 Å
3	19.152 °	4.63035 Å	16.168 °	5.47757 Å
4	19.710 °	4.50064 Å	19.987 °	4.43889 Å
5	20.049 °	4.42522 Å	24.339 °	3.65415 Å
6	20.977 °	4.23160 Å	26.810 °	3.32271 Å
7	22.117 °	4.01585 Å	31.768 °	2.81452 Å
8	22.424 °	3.96162 Å	32.716 °	2.73504 Å
9	22.935 °	3.87456 Å	38.548 °	2.33361 Å
10	23.531 °	3.77778 Å	41.268 °	2.18591 Å
11	23.772 °	3.73990 Å	43.507 °	2.07844 Å
12	24.397 °	3.64554 Å		
13	25.851 °	3.44372 Å		
14	26.845 °	3.31836 Å		
15	28.935 °	3.08326 Å		
16	29.524 °	3.02312 Å		
17	30.120 °	2.96465 Å		
18	31.820 °	2.81002 Å		
19	32.397 °	2.76129 Å		
20	32.734 °	2.73364 Å		
21	35.169 °	2.54971 Å		
22	35.631 °	2.51769 Å		
23	36.531 °	2.45774 Å		
24	38.553 °	2.33337 Å		
25	38.932 °	2.31152 Å		
26	41.280 °	2.18528 Å		
27	41.673 °	2.16557 Å		
28	42.606 °	2.12026 Å		
29	43.537 °	2.07709 Å		

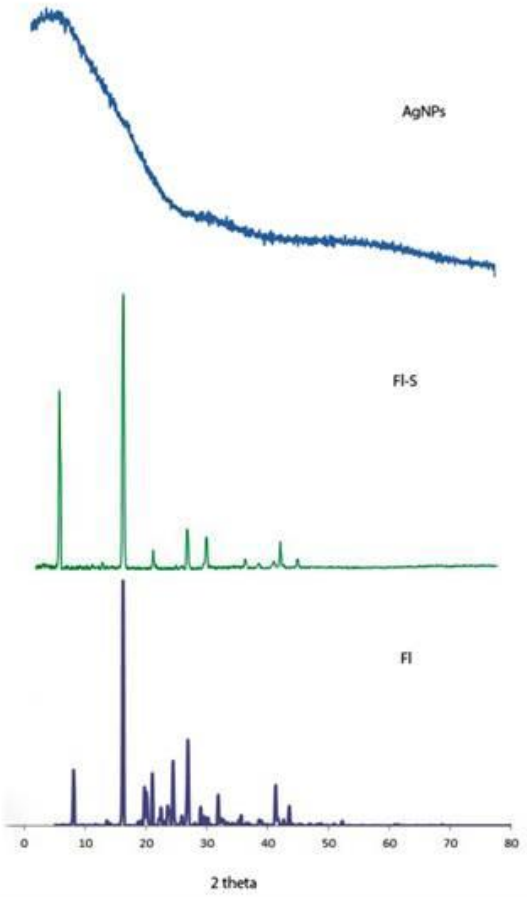


Fig. 1: XRD chart of florfenicol, florfenicol-silver nanocomposite and silver nanoparticles.

53.4, 78.6 and 146.9 cm^{-1} band observed in Raman spectrum were corresponding to lattice vibrations of fluorine crystal. The 225.7 cm^{-1} band showed in Raman spectrum may be accounted to υ(C-C-C). The 257.4, 325.8 and 360.1 cm^{-1} bands in Raman spectrum were attributed to δ(CC) aliphatic chains while the 473.97 cm^{-1} band in Raman spectrum may be assigned to υ(CH_3-S). In addition, the 630.1 cm^{-1} band in Raman spectrum can be accounted to υ(S=O), while the one at 720.4 cm^{-1} may be attributed to υ(C-S) aliphatic. 769.1 cm^{-1} band in Raman spectrum may be corresponding to υ(C-Cl). The bands at 816.1, 856.1, 879.95, 989.3 and 1302.4 cm^{-1} may be corresponding to υ (CC) alicyclic and aliphatic chain vibrations. The bands observed in the Raman spectrum at 1101.57, 1139.74 and 1196.11cm^{-1} may be accounted to υ(C=S) where the band at 1139.74 cm^{-1} was very strong Raman peak. Bands at 1598.24 cm^{-1} and 1680.4 cm^{-1} observed in Raman spectrum were accounted to υ(CC) and υ(C=N) aromatic ring chain vibrations, respectively. Strong Raman shift peaks at 2920.85 cm^{-1} and 2987.04 cm^{-1} can be assigned to υ(C-H) stretching mode of (CH_2) and (CH_3), respectively. Silver nanoparticles Raman spectrum illustrated very sharp three peaks at Raman shift range from 50 to 200 cm^{-1} (56.11, 98.1 and 146.2 cm^{-1}) which represented vibration of silver nanoparticles lattice. Raman shift bands at 550.92, 836.80, 950.30, 1095.61, 1322.58 and 1600.66 cm^{-1} represented vibration of AgO resulted from oxidation of silver nanoparticles by laser power. Florfenicol-silver nanocomposite showed sixteen Raman shift peaks at 36.69, 54.47, 147.01, 234.40, 462.15, 653.85, 764.36, 857.95, 1070.89, 1305.06, 1588.06, 2111.25, 2291.18, 2406.70, 2737.77 and 2930.56 cm^{-1}. There is no shift in Raman shift spectrum of florfenicol-silver nanocomposite than individual florfenicol and silver nanoparticles just overlap between them lead to merge Raman shift peaks together which created broad peaks.

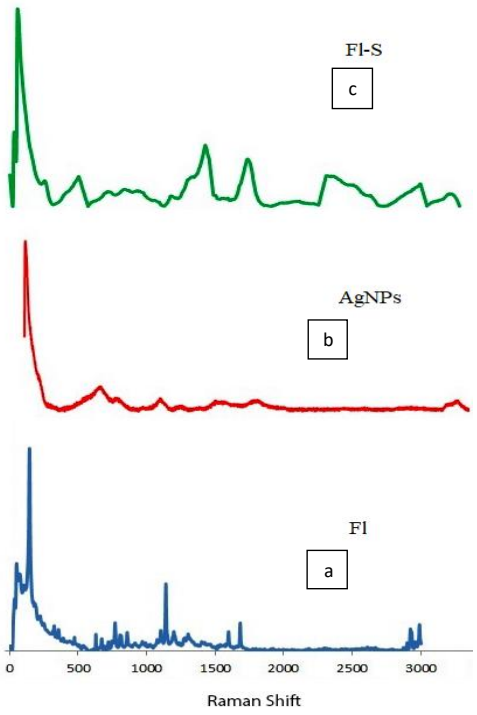


Fig. 2: Raman spectra of (a) florfenicol, (b) silver nanoparticles and (c) florfenicol-silver nanocomposite.

Microscopic

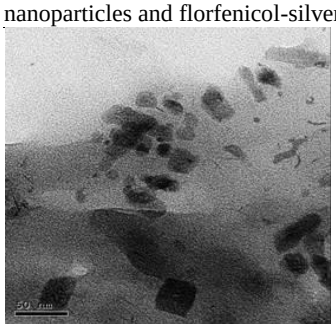
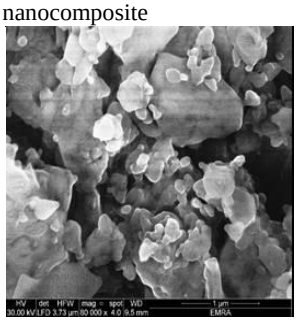
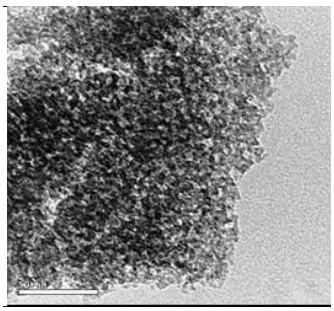
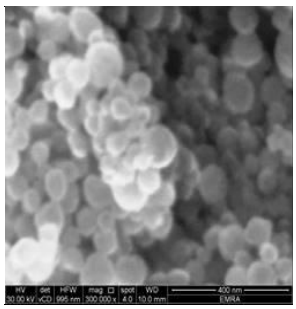
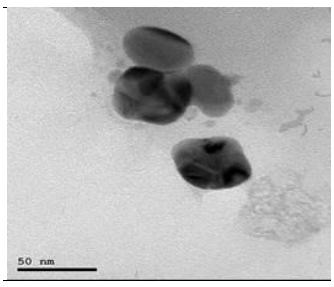
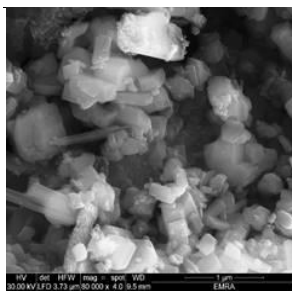
SEM and TEM data

3D SEM image and 2D TEM images for florfenicol, silver nanoparticles and florfenicol-silver nanocomposite were shown in table (3). SEM images illustrated the separated sheet structure of florfenicol which have sharp edge and thickness in nanosized form (about 20 nm) while TEM image illustrated sheet to cubic shape but the dominated shape was sheet. Silver nanoparticles have spherical shape with homogenous shape and size with size according to TEM image about 3 nm with spherical shape. SEM and TEM images of florfenicol-silver nanocomposite illustrated sheet of florfenicol loaded on its surface silver nanoparticles of spherical shape with size about less than 50 nm.

AFM data

AFM (atomic force microscope) measurement was just done for florfenicol-silver nanocomposite to confirm the shape, size and agglomeration obtained from TEM and SEM data. AFM images (Figure 3) illustrated sheet structure of florfenicol-silver nanocomposite (blue color) with maximum thickness less than 50 nm. Florfenicol-silver nanocomposite separated from each other and not tend to agglomeration or concentrated in certain area.

Table 3: 3D SEM and 2D TEM images for florfenicol, silver nanoparticles and florfenicol-silver nanocomposite

	
TEM image of florfenicol.	**SEM image of florfenicol.**
	
TEM image of AgNPs.	**SEM image of AgNPs.**
	
TEM image of florfenicol-silver nanocomposite.	**SEM image of florfenicol-silver nanocomposite.**

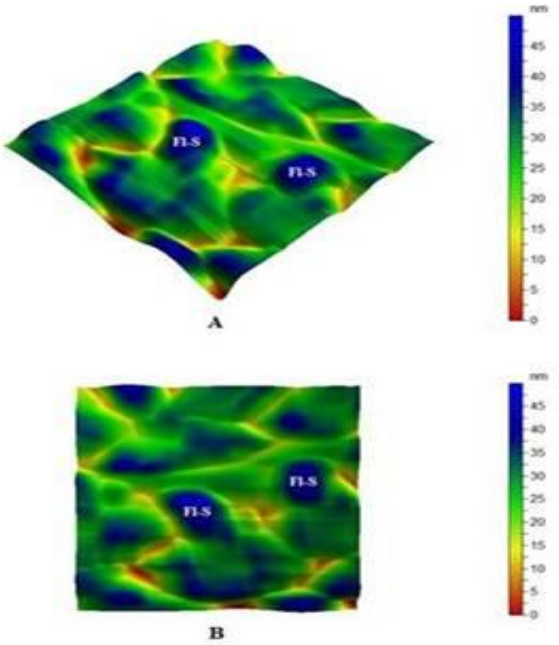


Fig. 3: AFM images of florfenicol-silver nanocomposite where A) 3D AFM image and B) top view one.

BET surface area measurement

BET surface area was measured for florfenicol, silver nanoparticles and florfenicol-silver nanocomposite to evaluate the chemical activity of florfenicol-silver nanocomposite than florfenicol and silver nanoparticles where the high BET surface area value lead to high chemical activity. BET surface area was found to be 13.3, 73.2 and 103.69 m^2/g for florfenicol, silver nanoparticles and florfenicol-silver nanocomposite, respectively. The highest BET surface area of florfenicol-silver nanocomposite lead to enhancement effect as anti-bacterial, antifungal and anti-cancer where high BET surface area increased contact with bacteria cell wall and the very high chemical activity of florfenicol-silver nanocomposite leads to increasing killing activity.

Zeta size and zeta potential

Zeta sizing and potential were measured for florfenicol and florfenicol-silver nanocomposite to determine the size and stability of them in aqueous media. Zeta sizing of florfenicol was found to be 30.44 nm while florfenicol-silver nanocomposite was 33.5 nm with zeta potential -14.1 and -18, respectively. The size matched with size obtain by TEM and AFM images and zeta potential values illustrated the good stability of both florfenicol and florfenicol-silver nanocomposite in aqueous media.

Antimicrobial assay using disc diffusion test

Results listed in Table (4) and Figures (4-7) showed that florfenicol-silver nanocomposite at the dose of 30 µg is more effective than its native form florfenicol and also reference standard positive. The average zone of inhibitions caused by the prepared nanocomposite were 28.3 mm, 24 mm, 27.3 mm and 24 mm compared to 17.7 mm, 16 mm, 18.7 mm and 13.3 mm of the native drug and 13 mm, 10 mm, 14.3 mm and 15 mm of the used positive reference standards against *E. coli*, *Salmonella typhymurium*, *Staphylococcus aureus* and *Staph.aureus* MRSA respectively.

Table 4: Average inhibition zone in mm.

	Average diameter of the inhibition zone (mm)		
	Florfenicol	Florfenicol-Silver nanocomposite	Positive reference standard
Escherichia coli (ATCC35218)	17.7 mm	28.3 mm	13 mm
Salmonella typhymurium (ATCC14028)	16 mm	24 mm	10 mm
Staph.aureus (ATCC29213)	18.7 mm	27.3 mm	14.3 mm
Staph.aureus MRSA (ATCC43300)	13.3 mm	24 mm	15 mm

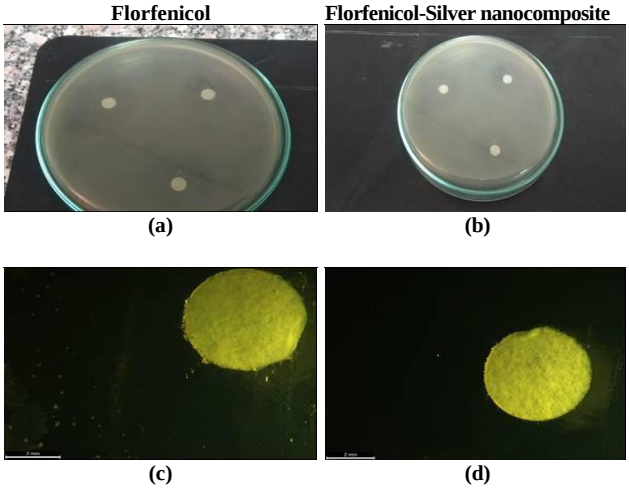


Fig. 4: Photographic images (a,b) and image by sterio microscope (a,b) show the zone of inhibition caused by florfenicol and florfenicol-silver nanocomposite against *Escherichia coli*.

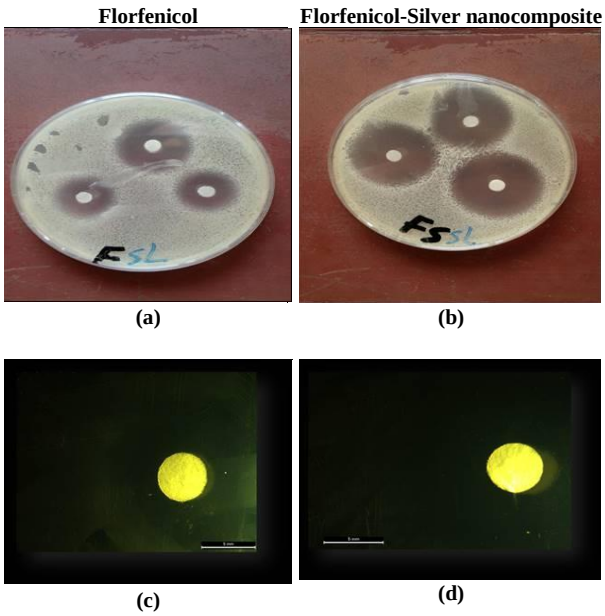
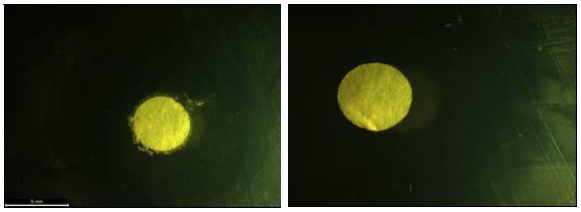


Fig. 5: Photographic images (a,b) and images by sterio microscope (a,b) show the zone of inhibition caused by florfenicol and florfenicol-silver nanocomposite against *salmonella typhymurium.*



(c) **(d)**

Fig. 6: Photographic images (a,b) and images by sterio microscope (a,b) show the zone of inhibition caused by florfenicol and florfenicol-Silver nanocomposite against *Staphylococcus aureus.*

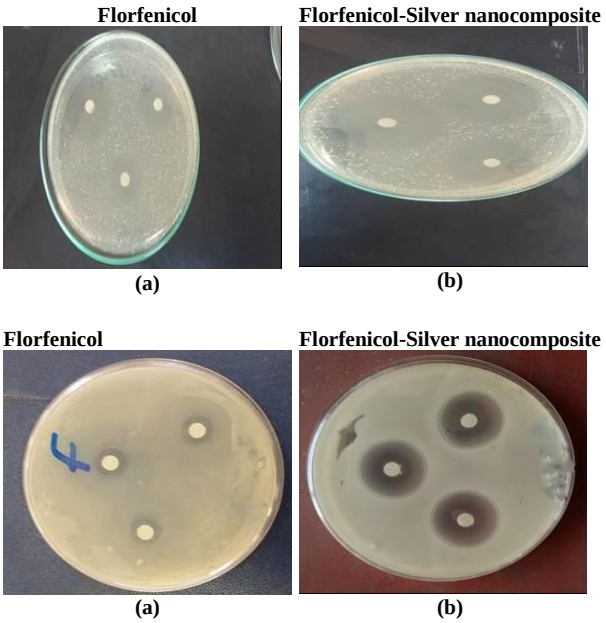


Fig. 7: Photographic images (a,b) show the zone of inhibition caused by florfenicol and florfenicol-Silver nanocomposite against *Staphylococcus aureus MRSA.*

From our results of antimicrobial activity of the prepared nanocomposite we proposed that silver nanoparticles at small dose makes a synergistic effect and potentiates the antimicrobial effect of florfenicol within the prepared nanocomposite against the tested gram positive and gram negative bacteria. This may be due to its activity against bacteria by attacking the respiratory chain and cell division of the microorganism resulting in cell death this mechanism was mentioned by Kim *et al.* (2013) and Youssef *et al.* (2019).

Conclusion

Nanotechnology is considered a leading new technology used to increase efficacy of antimicrobials and overcome the microbial resistance. This paper includes the preparation, characterization and in vitro antimicrobial activity of florfenicol silver nanocomposite in comparison with the native florfenicol antibiotic. Results of zeta size, zeta potential, XRD and Raman charts confirmed the formation of florfenicol-silver nanocomposite. TEM, SEM and AFM data illustrated spherical to sub spherical shape of silver nanoparticles on cubic to sheet shape of florfenicol with size less than 50 nm. Zone of inhibitions caused by the prepared nanocomposite against the tested strains were greater than that of the native florfenicol antibiotic and the used positive reference standards. According to the obtained results, our future studies will be focused on performing safety and toxicity studies, efficacy and pharmacokinetic studies of the prepared nanocomposite and the tested drug using animal model.

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