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Synthesis, Characterization and In vitro Antimicrobial Activity of Florfenicol-Chitosan Nanocomposite

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Abstract

In this paper, rapid, easy and cheap sonochemical method was described for synthesis of florfenicol-chitosan nanocomposite and to evaluate its anti-bacterial effect against *Escherichia coli* (ATCC35218), *Salmonella typhymurium* (ATCC14028) and *Staph. aureus* (ATCC29213). Florfenicol-chitosan nanocomposite was fully characterized for index, identification and morphology properties. Results of zeta potential for florfenicol-chitosan nanocomposite was -28 mV. Brunner-Emmett-Teller theory (BET) surface area was found to be 13.3, 73.2 and 103.69 m^2/g for florfenicol, chitosan nanoparticles and florfenicol-chitosan nanocomposite, respectively. Raman charts confirmed the formation of florfenicol-chitosan nanocomposite without any contamination. Transmission electron microscope (TEM), scanning electron microscope (SEM) and atomic force microscope (AFM) images and data illustrated spherical to sub spherical shape of florfenicol nanoparticles on sheet shape of chitosan with size less than 75 nm. Remarkable results of florfenicol-chitosan nanocomposite as anti-bacterial agent illustrated the power of nanotechnology. However, antimicrobial activity was screened where the zone of inhibitions caused by the prepared nanocomposite were 24.7 mm, 30.6 mm and 29.3 mm compared to 17.7 mm, 16 mm and 18.7 mm of the native drug against *E. coli*, *Salmonella typhymurium* and *Staphylococcus aureus*, respectively.

Keywords: Florfenicol; Chitosan nanoparticles; Florfenicol-chitosan nanocomposite; Antimicrobial activity; Microscopic techniques.

1. Introduction

Around 1970, nanotechnology was considered as a promising technology which was designed for preparation of unique materials their size ranged from one to hundred nanometers [1-3]. Also, it participated in many fields such as; agriculture, biology, and drug delivery [4 – 6]. Many articles and references approved that nanoparticles are more effective and beneficial than the bulk materials due to their large surface area, higher potency and bioactivity, In addition they are also characterized by controlled molecule size and excellent in drug delivery [4 – 17].

McMillan *et. al* [18] reported that the use of nanostructured products in veterinary medicine, have shown excellent results against multi-resistant microorganisms and bacterial strains.

Due to biocompatibility, biodegradability, and lower toxicity of chitosan it is recommended to be used in many applications and also considered as one of the most extensively used polymers in comparison with other biological polymers. Many literatures confirmed that synthesis of chitosan nanoparticles is

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mainly based on the ionic gelation method by the interaction of oppositely charged macromolecules, In the field of drug delivery system, chitosan nanoparticles are considered as unique carrier for poorly absorbed and less soluble materials [19 - 21].

There were some studies which confirmed the potential role of chitosan nanoparticles in antimicrobial therapy**.** Porras-Gómez ***et. al*** [22] studied the potential antimicrobial activity of ampicillin-loaded chitosan nanoparticles against one of the gram negative bacteria *Escherichia coli*. In addition, Carmona ***et. al*** [23] prepared chitosan nanoparticles loaded with florfenicol through the ionic gelation method in order to improve the efficiency and control the release of the florfenicol.

Florfenicol is used in veterinary medicine as a broad-spectrum antibiotic [24]. It does not result in aplastic anemia [4]. Florfenicol possess some disadvantages like poor water solubility in aqueous solutions [3]. Organic solvents are usually used in the preparation of the drug [25]. However this feature of the organic solvents in increasing the solubility of the drug, they result in more toxicity [26]. What's more, in certain mammals the half-life ($t_{1/2}$) of florfenicol is under three hours and an extra dose is needed for obtaining higher efficacy. From this way and along these lines, the advancement of new and novel formulations of the florfenicol will have a potential value [4, 27, 28].

Some literatures prepared florfenicol nanoparticle formulations. Song *et. al* [29] used silica nanoparticles for loading florfenicol and characterization techniques confirmed that the formed nanoparticle preparation showed a slower prolonged sustained release than the bulk native florfenicol. Wang *et. al* [30] used hot homogenization and ultrasonic technique for preparation of florfenicol-loaded solid lipid nanoparticle. The prepared nanoparticle formulation showed more antimicrobial activity than the bulk native drug against *Escherichia coli*.

The aim of our promising work was the preparation and characterization of florfenicol-chitosan nanocomposite. Also, studying the comparative in vitro antimicrobial activity of the prepared nanocomposite against some Gram positive and Gram negative bacteria and the native drug.

2. Experimental

2.1. Materials

All chemicals in this study are lab grade and used without any purification. Chitosan manufactured by Sigma Company. Florfenicol powder was supplied by Pharma Sweed Company.

2.2. Synthesis of chitosan nanoparticles

By ionotropic gelation method chitosan nanoparticles were synthesized according to method mentioned by Calvo ***et. al*** [31] in which the amine group of chitosan and tripolyphosphate was interacted by electrostatic interaction. However, 3 g chitosan dissolved in diluted glacial acetic acid (4 ml glacial acetic acid to 296 ml doubled deionized water) was added to 2g sodium tripolyphosphate in 200 ml doubled deionized water and stirring for 4 hours then putted at 40 ºC for 24 hours until whit gel was obtained. Finally, the sample was dried at 40 ºC in oven for 24 hours.

2.3. Synthesis of florfenicol-chitosan nanocomposite

Synthesis was carried out by sonochemical method in which the formation and collapse of microbubbles resulted from ultrasonic effect created extreme condition which forced florfenicol nanoparticles to be loaded to chitosan sheet structure. However, 0.1 g of florfenicol powder was added to 100 ml doubled distilled water and stirred at 1200 RPM at 45 ºC using magnetic stirrer for 30 minutes then 0.1 g of chitosan nanoparticles was added and continues stirring was extended for another 30 minutes. Finally, the mixture was left in ultra-sonic apparatus for nanocomposite formation under the following conditions (plus every 2 seconds at 85% amplitude power maximum temperature for 360 minutes).

2.4. Characterization

The aim of characterization of florfenicol-chitosan nanocomposite was used not only to determine physico-chemical properties but also to influence its anti-bacterial effect. Characterization was classified into three classes namely index, identification and morphology classes (Fig. 1). Composition class was done by raman spectroscopy which performed using Lab. RAM-HR Evolution Horiba Co. 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 100 X 100 nm, speed 0.5 inch/sec, I Gain = 0.5 and P Gain = 20 using contact mode). Index class was carried out to determine zeta potential (to confirm charge of the sample to know if it is colloidal or not) and zeta size which was also determined in this sector by nano Sight NS500 manufactured by Malvern, UK.

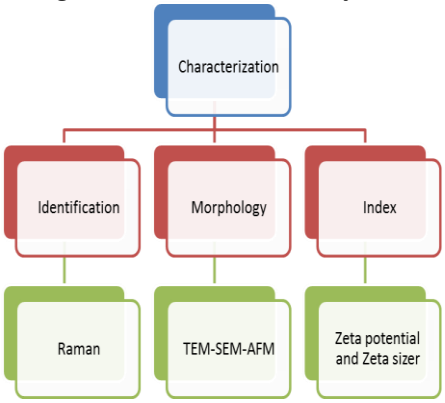


Fig. 1 The characterization tools of florfenicol-chitosan nanocomposite.

2.5. Antibacterial activity assay using disc diffusion method

Florfenicol-chitosan nanocomposite was tested for its antimicrobial activity against some 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 of 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 [32].

Paper discs (6 mm in diameter) were impregnated with 30 µL of the tested preparation with concentration of 1mg/ml and placed on Mueller Hinton agar plates, which were inoculated with test organisms**.** The plates were incubated at 37 °C for 24 hours and the diameters of the inhibition zones were measured. Additionally, for comparative purposes, polymyxin and kanamycin antibiotics were used as a reference standard. Each assay was performed in triplicate and repeated three times. The culture medium, incubation condition and reference standard used for each micro-organism were given in Table (1).

Table 1 Micro-organism's culture medium, incubation condition and reference standard.

Microbial type	Medium	Incubation condition	Positive reference standard
Escherichia coli **(ATCC35218)** ***Salmonella typhymurium*** **(ATCC14028)** ***Staph. aureus*** **(ATCC29213)**	Mueller Hinton Agar	37 °C for 24 hours	Polymyxin (disc loaded with 130 unit) Kanamycin (disc loaded with 30 µg)

3. Results and Discussion

3.1. Identification

3.1.1. Raman Spectra

To confirm the formation of florfenicol-chitosan nanocomposite, Raman shift measurement was carried out to chitosan nanoparticles and florfenicol-chitosan nanocomposite as shown in (Fig. 2). Raman spectrum of chitosan was characterized by 12 characteristic Raman shift peaks at 895.48, 1037.68, 1096.46, 1114.12, 1148.12, 1255.03, 1374.46, 1420.33, 1454.49, 1598.62, 2724, 2880.27 and 2921.60 cm^{-1}. Peaks at 895.48 and 1255.03 cm^{-1} represented vibration of C-H deformation plan where peaks at 1420.33 and 1454.49 cm^{-1} represented C-H deformation symmetry and asymmetry, respectively. Peaks at 1037.68 and 1148.12 cm^{-1} were attributed to vibration of C-O stretching while the bands at 1096.46 and 1114.12 cm^{-1} were accounted to C-O-C (ring) and C-O-C (ether), respectively. The 1374.46 and 1558.62 cm^{-1} bands represented vibration of C-N and N-H stretching vibrations, respectively. Finally, the most three Raman shift peaks characteristic to chitosan nanosheet were found at 2724, 2880.27 and 2921.60 cm^{-1} which accounted to υ(C-H) stretching mode of (CH_2) and (CH_3), respectively. Raman spectrum of florfenicol-chitosan nanocomposite illustrated characteristic peaks of chitosan and florfenicol. The 225.36 cm^{-1} band attributed to υ(C-C-C). The 257.44, 325.6 and 360.4 cm^{-1} bands were corresponding to δ(CC) aliphatic chains while the peak at 473.92 cm^{-1} assigned to υ(CH_3-S). The 630.13 cm^{-1} band found in the Raman spectrum may be accounted to υ(S=O), while the peak at 720.4 cm^{-1}

may be accounted to υ(C-S) aliphatic mode. The 769.1 cm^{-1} band in Raman shift may be assigned to υ(C-Cl). The bands at 817.4, 856.24, 878.88, 988.13 and 1303.14 cm^{-1} may be correlated to υ(CC) alicyclic, and aliphatic chain vibrations. The bands observed at 1101.37, 1139.64 and 1196.41cm^{-1} may be accounted to υ(C=S) while. Bands observed at 1600.25 and 1681.3 cm^{-1} were accounted to υ(CC) and υ(C=N) aromatic ring chain vibrations, respectively. Strong Raman shift peaks at 2921.77 and 2988.14 cm^{-1} were assigned to υ(C-H) stretching mode of (CH_2) and (CH_3), respectively.

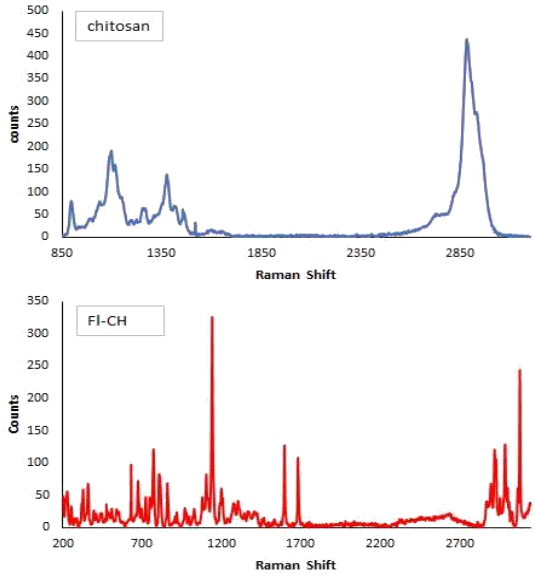


Fig. 2 Raman spectra of chitosan nanosheets and florfenicol-chitosan nanocomposite.

3.2. Scanning Electron Microscope (SEM) and Transmission Electron Microscope (TEM) Data

3D SEM image and 2D TEM images for florfenicol-chitosan nanocomposite were shown in (Fig. 3) and (Fig. 4). SEM images illustrated the separated sheet structure of florfenicol with sharp edge and thickness in nanosized form (about 25 nm) loaded into chitosan nanosheets which have thickness larger than florfenicol while TEM image confirm the SEM images where both of chitosan and florfenicol have nanosheet structure. SEM and TEM images of florfenicol-chitosan nanocomposite confirmed the formation of nanocomposite.

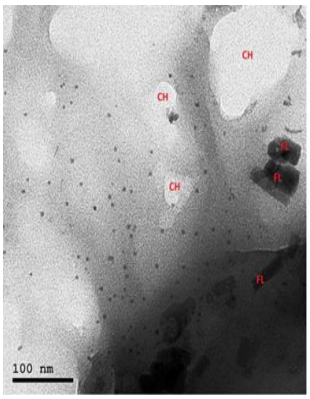


Fig. 3 TEM image of florfenicol-chitosan nanocomposite

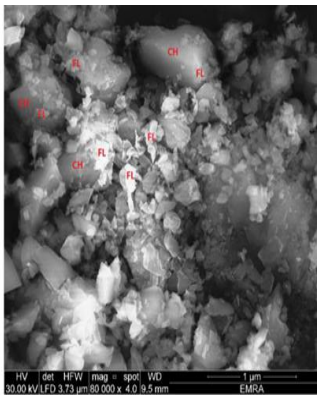


Fig. 4 SEM image of florfenicol-chitosan nanocomposite

3.3. Atomic Force Microscope (AFM) study

AFM measurement was done for florfenicol-chitosan nanocomposite to confirm the shape, size, concentration and agglomeration obtained from TEM and SEM data. AFM images as ashown in (Fig. 5) illustrated the sheet structure of florfenicol-chitosan nanocomposite (blue color) with maximum thickness less than 45 nm. It was clear from the figure that the florfenicol-chitosan nanocomposite was separated from each other and not tends to form agglomeration in certain area.

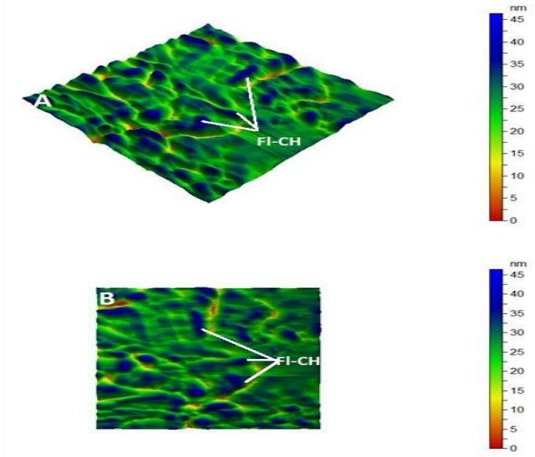


Fig. 5 AFM images of florfenicol-chitosan nanocomposite where A) 3D AFM image and B) top view one.

3.4. BET Surface Area Measurement

BET surface area was measured for native florfenicol, chitosan nanoparticles and florfenicol-chitosan nanocomposite to evaluate the chemical activity of florfenicol-chitosan nanocomposite in comparison with florfenicol and chitosan 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, chitosan nanoparticles and florfenicol-chitosan nanocomposite, respectively. The highest BET surface area of florfenicol-chitosan nanocomposite can be assigned to the enhancement effect as anti-bacterial, antifungal and anti-cancer. The high BET surface area increased contact between florfenicol-chitosan nanocomposite and bacterial cell wall and resulted in increasing the killing activity of the prepared nanocompoite.

3.5. Zeta size and zeta potential

Zeta size and potential were measured for florfenicol-chitosan nanocomposite to determine its size and stability in aqueous media. Particle size of florfenicol-chitosan nanocomposite was found to be 67.22 nm with zeta potential -28 mV, respectively. The size obtained matched with size given by TEM, SEM and AFM images and zeta potential values illustrated the good stability of florfenicol-chitosan nanocomposite in aqueous media.

3.6. Antimicrobial assay using disc diffusion test

Results listed in (Fig.7) showed that florfenicol-chitosan nanocomposite at the dose of 30 µg is more effective than its native form florfenicol and also positive reference standard. Images presented in (Fig.8), (Fig.9) and (Fig.10) showed zone of inhibitions caused by the prepared nanocomposite were 24.7 mm, 30.6 mm and 29.3 mm compared to 17.7 mm, 16 mm and 18.7 mm of the native drug and 10 mm, 10 mm and 14.3 mm of the used positive reference standard against *Escherichia coli, salmonella typhymurium and Staphylococcus aureus*, respectively.

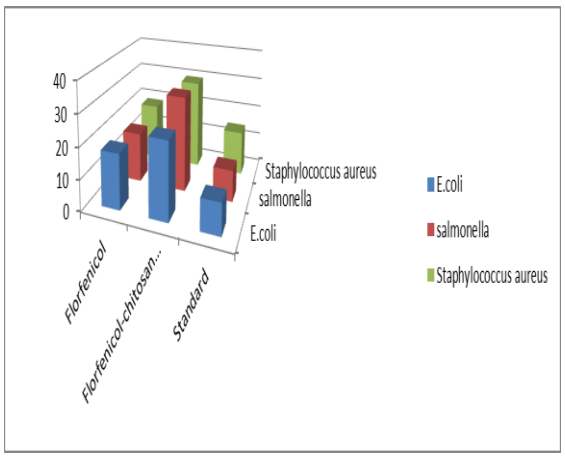


Fig. 7 Antimicrobial activity of florfenicol, florfenicol-chitosan nanocomposite and standard against different organisms.

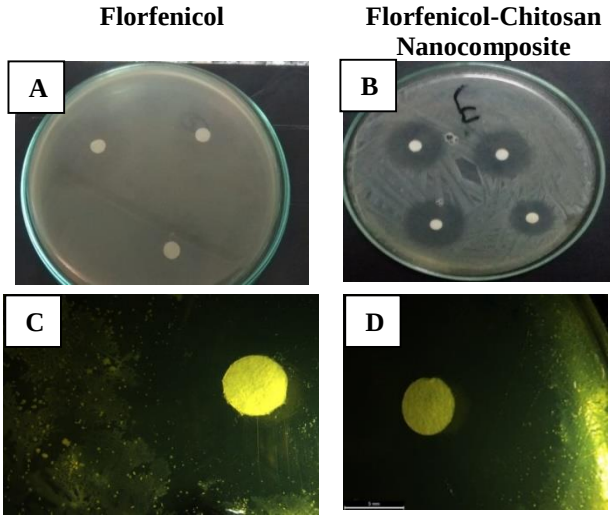


Fig. 8 Photographic images (a,b) and stereo-microscopic images (c,d) showed inhibition zone caused by florfenicol and florfenicol-chitosan nanocomposite against *Escherichia coli.*

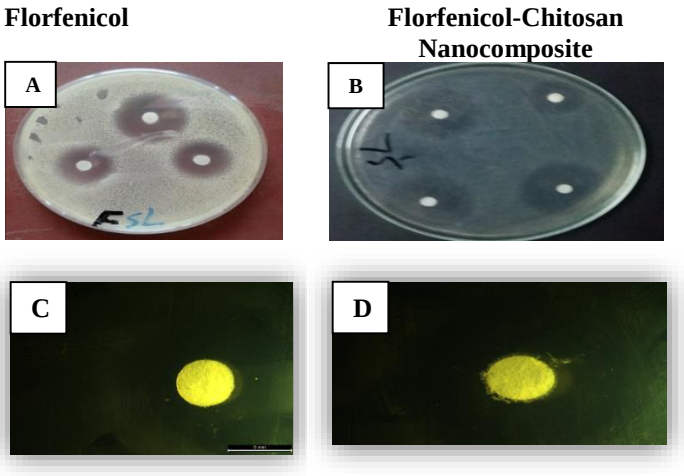
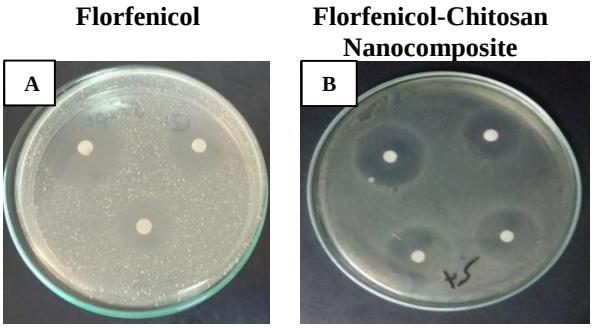


Fig. 9 Photographic images (a,b) and stereo-microscopic images (c,d) showed inhibition zone caused by florfenicol and florfenicol-chitosan nanocomposite against *salmonella typhymurium*.



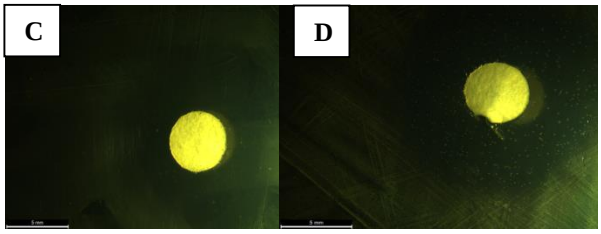


Fig. 10 Photographic images (a,b) and stereo-microscopic images (c,d) showed inhibition zone caused by florfenicol and florfenicol-chitosan nanocomposite against *Staphylococcus aureus*.

Florfenicol is a broad-spectrum antibiotic used for treatment of different diseases in veterinary medicine. However the feature of this antibiotic it posses some disadvantages like bacterial resistance, toxicity, poor water solubility and low disposal half-time. From this point of view, the advancement of florfenicol novel preparations will have a promising value.

Youssef *et. al* [3] prepared and characterized florfenicol-silver nanocomposite by the sonochemical method and investigated the antimicrobial efficacy of the prepared nanocomposite against some bacterial strains. The results showed that the prepared nanocomposite at the dose of 30 µg is more effective than its native florfenicol against *Escherichia coli*, *Salmonella Typhimurium*, and *Staphylococcus aureus*. They mentioned that the best antimicrobial activity may be attributed to the ability of the nanocomposite to attack the respiratory chain and cell division of the microorganism resulting in cell death.

In our study, chitosan nanoparticles were selected to be incorporated within the nanocomposite as it was reported to be safer and possess a potential antimicrobial and perfect for drug delivery with low toxicity [4]. No previous literatures studied the antimicrobial activity of the florfenicol-chitosan nanocomposite. The potential *in vitro* antimicrobial activity of the prepared nanocomposite may be attributed to the results of TEM, SEM and AFM which confirmed the spherical to sub spherical shape of florfenicol on sheet shape of chitosan with size less than 75 nm. The formed polycationic nanocompoite might have high affinity to interact with the negatively charged bacterial surface. Also large surface area facilitated their tight absorption to the surface of bacteria leading to disruption of the bacterial membrane and leakage of intracellular compounds and bacterial cell death.

3.7.1. Mechanism of bacterial inhibition of the prepared nanocomposite

Despite the fact that there is some discussion in the writing about the overall impact of nanoparticles on the sort of microscopic organisms, this examination is in accordance with reports that recommend that Gram negative microorganisms are progressively affected by polymeric and metal based materials [33 - 34]. The structure of the outer surface of the Gram-negative bacteria is differing from that in Gram-positive one. The peptidoglycan layer in gram negative bacteria is thinner (from 2 to 3 nm) while in Gram-positives is about 30 nm [35].

Antimicrobial mechanism of florfenicol-chitosan nanocomposite is still unknown; the direct electrostatic forces between the nanocomposite and bacterial cell surfaces causes' damage of membrane after the bacterial cell membrane damage and then the nanocomposite become able to permeate into it [36].

An interaction between bacteria and florfenicol-chitosan nanocomposite is supposed to course through the hydrogen bonding of active sites of protein (COOH and NH_2). Direct contact of florfenicol-chitosan nanocomposite with cell membranes, resulted in destructing bacterial cell integrity.The attachment of florfenicol-chitosan nanocomposite to bacterial cell walls and subsequent release of florfenicol to the bacterial cytoplasm was described as the mechanism of action. Florfenicol was attached to the biomolecules in the bacterial cell though electrostatic interaction [37].

Florfenicol chitosan nanocomposite was diffused inside the microbial cell may result in cell death. The antibacterial activities of florfenicol-chitosan nanocomposite against Gram-positive and Gram-negative bacteria were studied in this article. These bacterial strains have chemical composition and different cell wall structures [38].

4. Conclusion

Preparation of antibiotics loaded on nanoparticles in order to increase efficacy and overcome the microbial resistance became nowadays the interest subject of many researchers. Florfenicol-chitosan nanocomposite was prepared by sonochemical method and characterized using different techniques. In vitro antimicrobial study was performed by disc diffusion technique. According to the raman chart, florfenicol-chitosan nanocomposite was formed in pure state. Microscopic data (TEM, SEM and AFM) concluded the spherical to sub spherical shape of florfenicol nanoparticles on sheet shape of chitosan with size less than 75 nm. It was found that zeta size of florfenicol-chitosan nanocomposite was 67.22 nm with zeta potential of -28 mV, respectively. Zone of

inhibitions caused by the prepared nanocomposite were found to be 28.3 mm, 24 mm and 27.3 mm compared to 17.7 mm, 16 mm and 18.7 mm of the native drug against *E. coli*, *salmonella typhymurium and Staphylococcus aureus*, respectively. According to the results obtained, the coming research will be focused on performing the *in vivo* studies which will include; comparasion of the efficacy the prepared nanocomposite and the tested drug in infected animals. Comparasion of the pharmacokinetic activity. In addition, In vivo studying of the safety and toxicity of the prepared nanocomposite.

Conflicts of interest

There are no conflicts of interest to declare. The corresponding author is ready to provide any detailed data upon request.

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