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Impact of Chitosan Nanoparticles Loaded Oxytetracycline Hydrochloride for Drug Delivery Against *Flavobacterium Columnare* Isolated From Common Carp (*Cyprinus Carpio*L.)

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Abstract

This present study investigates a new method for oxytetracycline (OTC) application through the use of Chitosan nanoparticles (ChNPs) as drug delivery, used the ionic gelation method for preparation of chitosan nanoparticles and it's loaded. Also characterized the properties particle size, shape, encapsulation efficiency, and antibacterial activity against F.columnare isolated from common carp. The formulations are spherical.The diameter of chitosan nanoparticles size varying from 10-15 nm and chitosan nanoparticles loaded oxytetracycline(ChNPs-OTC) in size about 20 nm. With high encapsulation efficiency ranging from 99.4% to 99.8%. Antibacte¬rial activity was in vitro against Flavobacterium columnare using a good diffusion method, 5 concentrations of ChNPs-OTC (20,15,10,5, and 2.5 µg/ml) with 20ug/ml of blank oxytetracycline as control positive. The higher inhibition zone was recorded in ChNPs-OTC with higher concentration. These results suggest that ChNPs-OTC show possible using the delivery of drugs and improved treatment effectiveness for bacterial fish diseases.

Keywords: *ChitosanNanoparticle, Oxytetracycline, Ionic gelation, Encapsulation Efficiency*

I. Introduction

Nanotechnology is a study of the tiny structure and modification of matter on a scale of atoms and molecules to construct several novel supplies. Procedure and devices use nanoscale particulate matter, so it includes the production of small-scale components from 1 to 100 nm (1 nm=10^{-9} m) and on the nanoscale, materials begin to create their novel physical, chemical, and biological highly developed properties. Which has many usages in areas destinations including biochemistry, electric power as well as information systems, weather, Agriculture, Water

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Cleaner, biomedical applications, and many more. Solano et al. (2015). Many forms of nanoparticles have been used in medicine Jong and Borm,(2008), for example nanospheres. Along with its low size and high diameter, a certain compound may be dispersed on the nanostructured surface to allow the distribution of drugs. Nanospheres are also used to repair tissue. (Ramalingam et al., 2013).

Polymer nanoparticles, like ChNPs, for drug delivery through different routes of administration Wang et al. (2013). The nanoparticles have been used to deliver the drugs into the cells with negligible side effects Scott, (2005). Nanoparticles have gained as the delivery of drugs significant attention due to its small size, then they are capable of crossing biological membranes, it seems to have a large amount of total area to volume ratio, thus it leads to increased reactivity with other ligands and materials. Especially, chitosan nanoparticles and PLGA nanoparticles are being tested as nanoparticles for drug delivery in fish medicine. (Wang et al., 2011).

ChNPs seem to be biodegradable, stable, cheap, low toxic, good biocompatibility as well as easy to conduct also created from fully biocompatible and biodegradable natural polymer chitosan and are often approved by GRAS. Pangestuti and Kim,(2010). Due to the extreme wider surface area of the ChNPs, which can be adsorbed strongly on top of the surface of the cells of the microbes to destroy the membrane, this would lead to the outflow of intracellular components and thus kill the bacterial cells. Avadi et al. (2004); Abdel-Fattah et al. (2014). Chitosan nanoparticles were used to enhance their transfer efficiency in cells as drug carrier and gene carrier, as recorded in many studies (Chopra and Roberts, 2001; Csaba and Alonso, 2014)

In addition to the function of chitosan in attracting inflammatory cells, especially neutrophil, in early stages, neutrophilia was accompanied by an increased phagocytosis index E'atelaf, (2015). Nanostructures drug delivery systems may improve treatment effectiveness by increasing the concentration of antibiotics in the microbes without increasing the dose of antibiotics administered. Azhdarzadeh et al. (2012). Also, ChNPs are revealed to designate a first-class adjuvant used for vaccine carriers (Nascimento et al. (2014); Ragelle et al. (2014). Oxytetracycline hydrochloride (OTC-HCl) is among the most generally recognized antibacterial agent used in fisheries. Ragelle et al. (2014); Erdogdu, (2012), OTC-HCl is prescribed within the diet to control carp infections (Darwish et al. (2002); Thomas and Goodwin, (2004), in this study the ChNPs samples were prepared through ionic gelation method via sodium tripolyphosphate only as cross-linker Vimal et al. (2013). OTC content in Ch-NPs was calculated using a completely validated HPLC process (Morakul et al. (2014); Amini and Ahmadiani, (2005).), in vitro antibacterial activity also assessment.

II. Materials and Methods

1-Materials

Oxytetracycline hydrochloride, chitosan, sodium tripolyphosphate, phosphate-buffered saline, acetic acid, Cytophaga Agar, De-ionized water, and distilled water. All other chemicals were obtained from commercially from AL-Bashir Scientific Bureau/Iraq. Flavobacterium columnare was taken from the Department of fish diseases College of Veterinary Medicine/Baghdad University.

2-Preparation of chitosan nanoparticles

By ionic gelation process, the ChNPs were prepared from chitosan using sodium tripolyphosphate to cross linker Vimal et al. (2013), and for producing of a homogeneous chitosan solution, approximately 1.5 g of chitosan disbanded throughout 200 ml of 2% acetic acid, the whole mixture was taken under the magnetic stirring procedure for around 20 minutes. In addition to the above-prepared chitosan solution, 0.8 g of sodium tripolyphosphate diluted in 107 ml of conductivity water was applied drop wise then stirring very well for around Thirty minutes to achieve stabilization. A milky coloured emulsion of chitosan nanoparticles appears the ionic cross-linking of sodium tripolyphosphate and chitosan solution was formed. After achieving balance, the suspension was established in the conditions mentioned above.

3-Preparation of ChNP-OTC

Two concentrations of oxytetracycline hydrochloride In distilled water dissolved and added to the ChNPs solution of a percentage. 1:1 and 1:0.5 ChNPs to OTC (2 formulations) under stirring for 20 min. also, this resulting suspension then was left under ultrasonication for 45 minutes.Then lastly stirring for another 20 minutes, to obtain a final concentration of antibiotics 3.75 mg/ml and 1.875 mg/ml (Jain and Banerjee, 2008; Du *et al*.(2009).

Formulation 1: (ratio 1: 1 ChNPs to OTC)

- 0.75gof oxytetracycline hydrochloride (99% purity) added to 200 ml final nanoparticles solution.
- This solution becomes contains 3.75 mg per ml
- Total drug added = 3750 µg/ml

Formulation 2: (ratio 1: 0.5 ChNPs to OTC)

- 0.375g oxytetracycline hydrochloride (99% purity) added to 200 ml final nanoparticles solution.
- This solution becomes contains 1.875 mg per ml
- Total drug added = 1875 µg/ml

4-Characterization of chitosan nanoparticles loaded oxytetracycline

a-Transmission Electron Microscopy(TEM)

Two samples were sent for the examination of TEM, The first sample is chitosan nanoparticles, and the second, the sample was chitosan nanoparticles loaded oxytetracycline hydrochloride. The samples were examined using a JEOL.JEM- 1200EXII electron microscope.

b-Encapsulation Efficiency (EE%)

OTC content in ChNPs was calculated using a completely validated HPLC process (Amini and Ahmadiani,(2005); Morakul et al.(2014) , two formula was sent to the assessment of encapsulation efficiency to Veterinary Directorate -Department of biology and medical supervision. The first formulation of ChNPs-OTC ratio was 1:1 and the second formula was 1:0.5 ChNPs-OTC. Besides, send a standard oxytetracycline hydrochloride sample used in the experiment to be evaluated. Then, specimens of each formulation were injected with appropriate dilution into the HPLC column.

Encapsulation efficiency (EE %) in the ChNPs were calculated according to the Equation (Wang *et al*.,2008; Meng*et al.*,2011).

$$\text{Encapsulation efficiency}=\frac{(\text{Total drug content})-(\text{free drug content})}{\text{Total drug content}}\times 100$$

c-Antibacterial activity

Minimum Inhibitory Concentration (MIC)

Well diffusion method:

10 ml tube of Hsu-shotts broth were inoculated with the *F.columnare* and incubated at 25°C for overnight. 0.6ml of the broth culture of bacteria was added to 60ml of Moler Hinton agar by a sterile pipette, which was cooled at 45C°. Prepared by mixing well and put on a sterile Petri dish. It was allowed to set and harden the agar. The appropriate number of holes were cut and use a sterile cork borer to ensure proper peripheral and central distribution of the holes (Bohloli,2017).

Oxytetracycline hydrochloride was used in comparison with the antibacterial activity of Chitosan nanoparticles loaded oxytetracycline hydrochloride in different doses. Oxytetracycline hydrochloride 20ug/ml distilled water was placed inside the central hole as control +. Inside each peripheral holes placed different dose of freshly prepared ChNPs-OTC, UV light-sterilized for ten min. (Lee et al., 2012). The particles were then re-suspended in a volume of sterile water sufficient to achieve final Chitosan nanoparticles loaded oxytetracycline hydrochloride concentration is (20, 15,10,5 and 2.5 ug/ml w/v) and the peripheral holes gave numbers from 1 to 5 respectively. The plates were left at room temperature for 2 hours to permit the dispersion of the sample and the incubate face upward on 25 ° c for 24 hrs. The zones' diameter of inhibition was calculated with a measuring ruler. All tests were performed in triplicate.

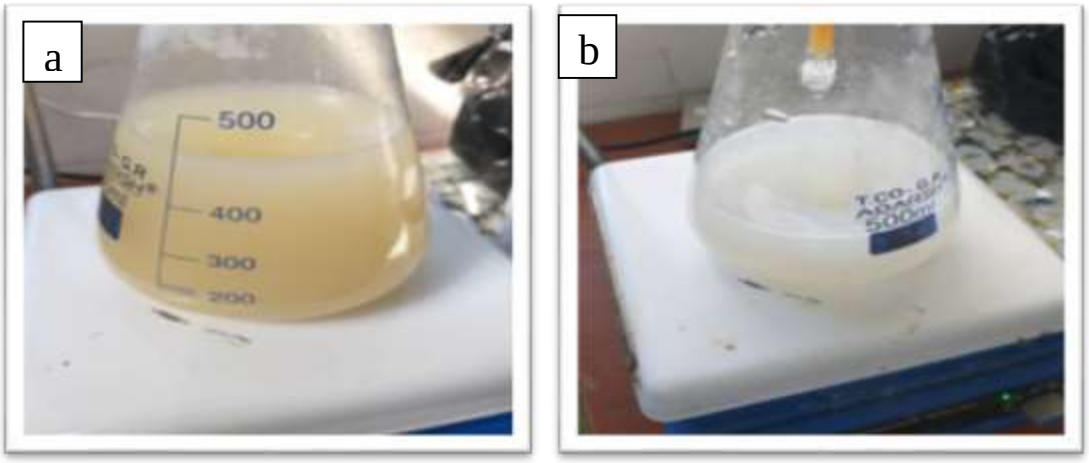
Statistical Analysis:

The program Statistical Analysis Framework- SAS (2012) has been used to determine the effect of various groups in parameters of the test. The least significant difference – LSD test (Variation-ANOVA Analysis) was used in this study to allow significant comparisons between measures.

III. Results

Nanoparticle Characterization

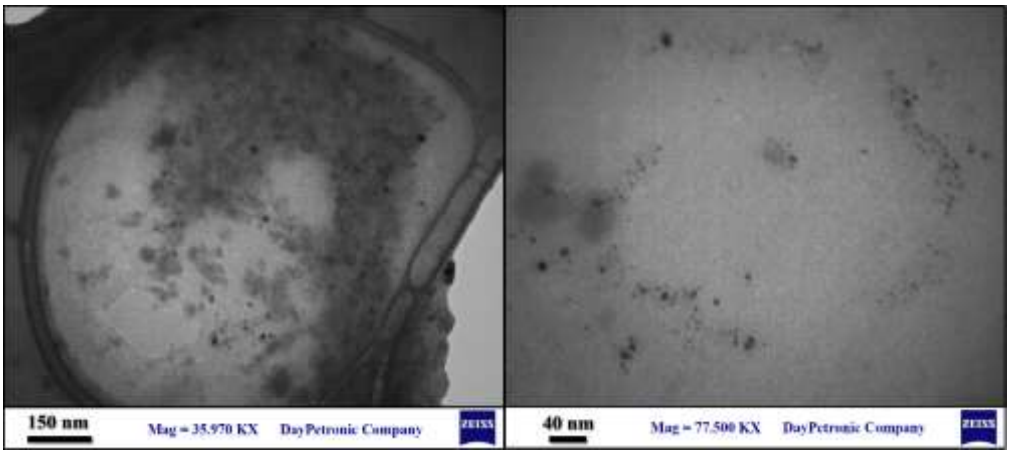
ChNPs are done based on an ionic gelation interaction between chitosan positively charged and tripolyphosphate negatively charged at room temperature as well as a milky coloring solution like the appearance of ChNPs, which showed in figure (1 A&B). The connection can be managed by chitosan - tripolyphosphate charge density, which depends mostly on solution ph. and ultra-sonication time.



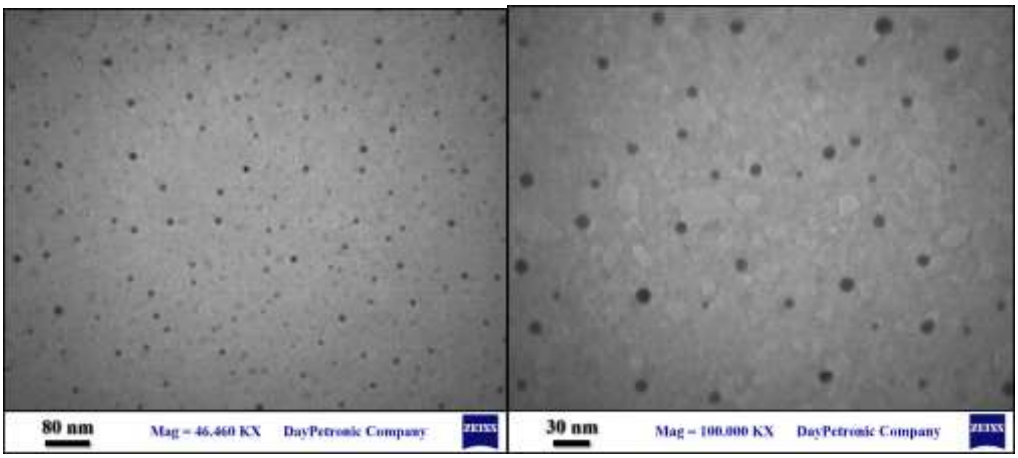
Figure(1 A&B)Color converted into a milky emulsion of chitosan nanoparticles.

Transmission electron microscopy (TEM)

TEM images have shown the morphological properties and surface appearance of chitosan nanoparticles and chitosan nanoparticles loaded oxytetracycline hydrochloride (ChNP-OTC). The nanoparticular shapes are smooth surfaces and almost spherical in shape. The diameter of chitosan nanoparticles size varying from 10-15 nm and chitosan nanoparticles loaded oxytetracycline(ChNPs-OTC) in size 15-20 nm as shown in figure (2C&D).



(a)



(b)

Figure 2(a): The chitosan nanoparticleshave a spherical shape, smooth surface and the size about 10-15 nm.(b): chitosan nanoparticles loaded oxytetracyclin hydrochloride and the size about15-20 nm.

Encapsulation Efficiency (EE%):

The oxytetracycline drug contents and encapsulation effectiveness (EE%) were performed using the previously validated HPLC process, and the results are presented in figures 3 (A, B & C). Profits and higher of the encapsulated drug were obtained for chitosan nanomaterials for both formulas. The Encapsulation Efficiency was in the range of 99.4% to 99.8 % of formulation1 and formulation2, respectively. High encapsulation of OTC in NPs indicated efficient loading of the drug. The absolute recoveries of OTC were determined by direct comparison of peak area form standard versus sample.

Figure3.a. A typical chromatogram of a 100 µg/ml oxytetracycline (OTC) standard solution.

Figure3.b. A typical chromatogram of a 22ug/ml in Formulation 1

Figure3.c. A typical chromatogram of a 3ug/ml in Formulation 2

The encapsulation efficiency was determined after the reading of the filtered samples in the HPLC, performed and calculated:

$$\text{EE\% F1}=\frac{(3750\text{ug/ml})-(22\text{ug/ml})}{3750\text{ug/ml}}\times 100 = 99.4\ \%$$

$$\text{EE\% F2}=\frac{(1875\text{ug/ml})-(3\text{ug/ml})}{1875\text{ug/ml}}\times 100 = 99.8\%$$

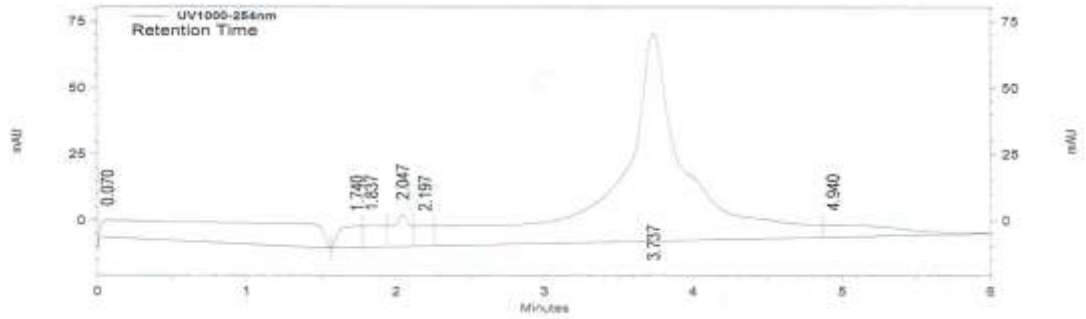


Figure (3.a): A typical chromatogram of a 100 µg/ml oxytetracycline (OTC) standard solution

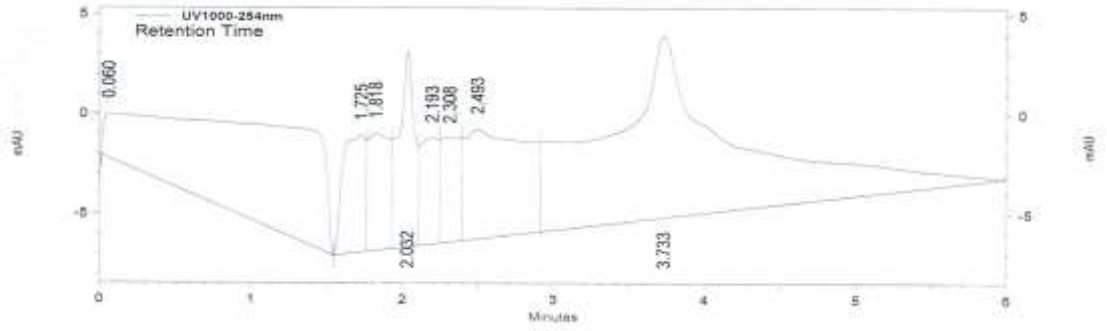


Figure (3.b): A typical chromatogram of a 22ug/ml in Formulation 1

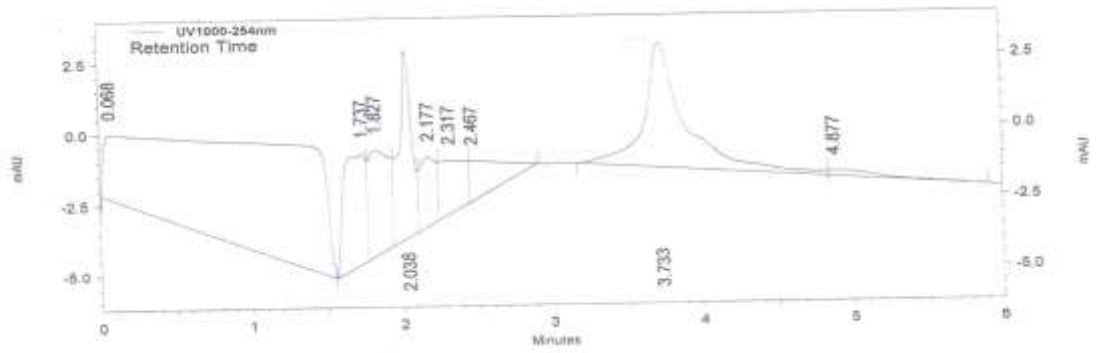


Figure (3.c):A typical chromatogram of a 3ug/ml in Formulation 2 .

Antibacterial activity

Results of the inhibition zone values for ChNPs-OTC against *F.columnare* are presented in Figures (4.A&B), Table and Figure (4). The inhibition zones were reported in millimeter (mm). CNP-OTC in concentration 20,15 and 10 ug/ml revealed a significant increase ($P<0.05$) showed high antibacterial activity against bacteria, which is higher than the same dosage in the control positive, and no inhibition control negative, while the ChNP-OTC in concentration 2.5 µg/ml revealed the less inhibition zone (8.06mm).

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Table (4.) : Effect of Compound concentration Growth Inhibition (mm)

Compound concentration	Mean ± SE of Growth Inhibition (mm)
Control(+) OTC 20 µg/ml	14.13 ± 0.17 e
ChNP-OTC 20 µg/ml	31.06 ± 0.24 a
ChNP-OTC 15 µg/ml	26.26 ± 0.14 b
ChNP-OTC 10 µg/ml	21.96 ± 0.24 c
ChNP-OTC 5 µg/ml	15.36 ± 0.22 d
ChNP-OTC 2.5 µg/ml	8.06 ± 0.14 f
LSD value	0.612 *
Means having with the different letters in same column differed significantly. * (P≤0.05).	

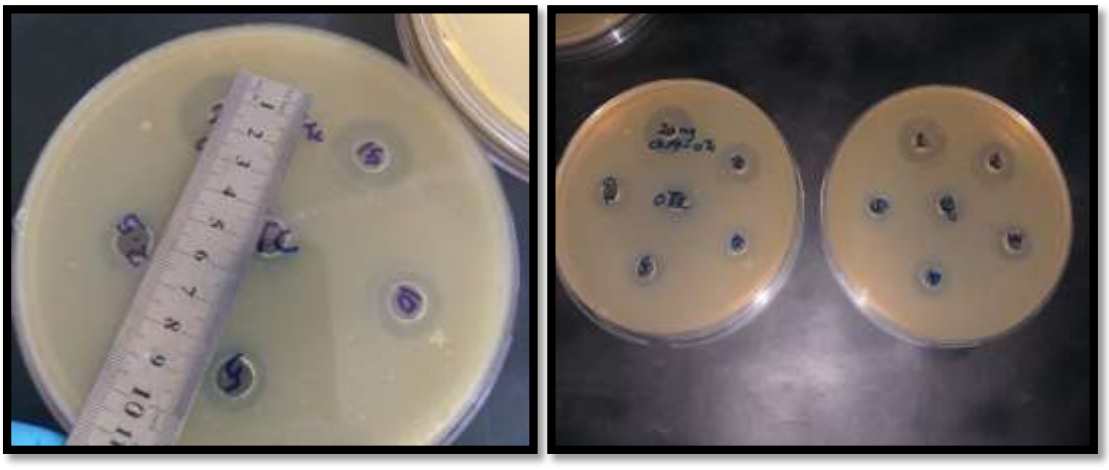

Figure 4. (a&b) Inhibiton effect of ChNPs-OTC against *F.columnare* by well diffusion methods .

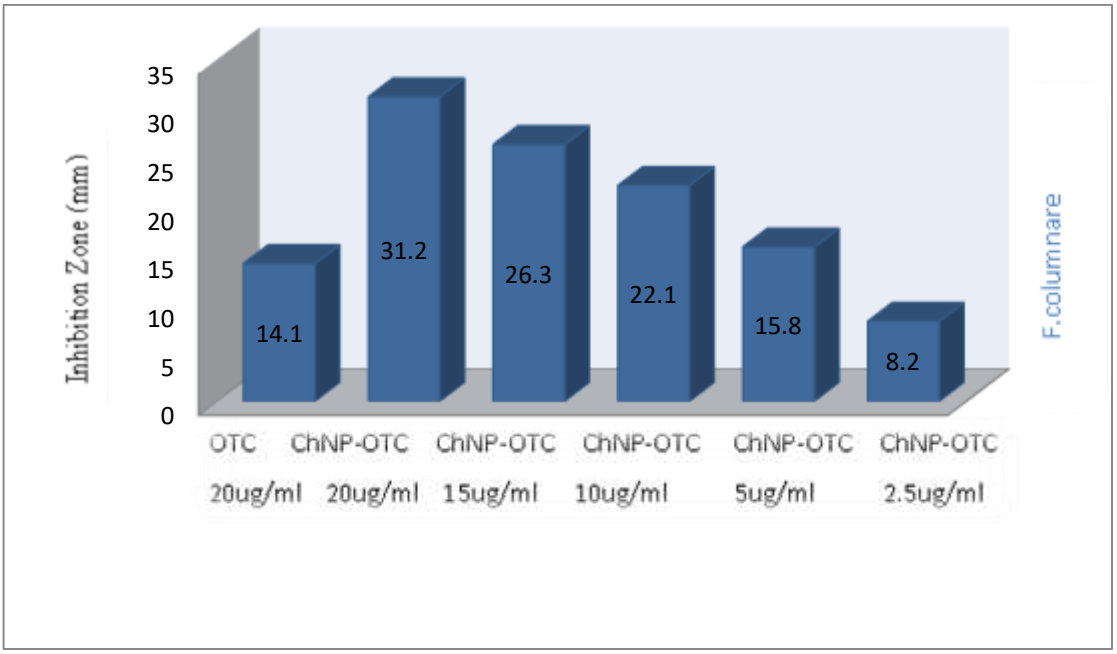

Figure (4.): Graph Diameter of inhibition zone (mm) caused by antimicrobial activity of ChNPs-OTC against *F.columnare* .

IV. Discussion

1-Characterization of ChNPs and ChNP-OTC:

In this study, we have synthesized and optimized Chitosan nanoparticles successfully loaded with oxytetracycline hydrochloride. On comparing the TEM micrograph details of chitosan nanoparticles loaded oxytetracycline with chitosan nanoparticles, it was observed that the nanoparticles of Chitosan have a relatively rough surface with an irregular shape. So many researchers also stated the spherical shape of chitosan nanoparticles made from chitosan, this result agrees with (Ghadi et al., 2014).

The ionic gelation system was successfully prepared. The ChNPs and ChNPs-OTC particles were smallish, with an average diameters10-15 nm and 15-20 nm respectively, in the TEM test The two-particle classes were smooth-edged spherical. The particles loaded on the drug were smaller than the free ones. The results agree with other results. (Gan et al.,2005; Alishahi et al.,2011).

2-Encapsulation Efficiency (EE%):

High amounts of OTC contribute to increased EE% and maximum drug losses. This would be due to the fact that increased drug concentration implies a larger EE%, also nanoparticle can hold with an increased loss of drugs outside the nanomaterial. This compatible with Muhamad et al. (2014). The result of our study the Encapsulation Efficiency was in the range of 99.4% to 99.8 % of formula1 and formula2 respectively. The high encapsulation efficiency of chitosan nanoparticles loaded oxytetracycline hydrochloride indicated the efficient

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loading of the drug. Also, Cover et al. (2012). reported the highest encapsulation efficiency of doxycycline loaded chitosan nanoparticles which were 69%.

3-Antibacterial activity:

Those nanoparticles of smaller sizes have strong cell membrane contacts, compared to bigger particles, due to the small particle endocytosis. The effectiveness of the particulate-drug delivery systems might well be increased by tiny particulate matter, and the use of small particles can also improve bioavailability and increase the efficacy of drugs that have been obtained by (Wardani and Sudjarwo,2018; Khanmohammadi et al.,2015).

All this agreed with our study where we recorded the high antibacterial activity of chitosan nanoparticles loaded oxytetracycline against F.columnare bacteria on well diffusion agar. CNPs-OTC in dose 20ug/ml showed significant ($p<0.05$) inhibitory effect against F.columnare when compared to unencapsulated oxytetracycline hydrochloride as a positive control. This high dose showed significantly ($p<0.05$) inhibitory effect on F.columnare compared to other doses and positive control at the same dosages.

V. Conclusion

ChNP-OTC was synthesized and characterized to investigate characteristics that may enhance the delivery and potency of drugs. Two types of nanoparticles were already constructed to different ratios of antibiotic particle sizes in TEM test ranged from 10-15 nm (ChNPs) however from 15-20 nm (ChNP-OTC). All these formulations of ChNPs-OTC were spherical in shape, with encapsulated efficiency of ranged from 99.4% to 99.8% in formula 1 and 2 respectively. Recorded the high antibacterial activity of chitosan nanoparticles loaded oxytetracycline against F.columnare bacteria on well diffusion agar.

CNPs-OTC in dose 20ug/ml showed significant ($p<0.05$) inhibitory effect against F.columnare when compared to unencapsulated oxytetracycline hydrochloride as a positive control, As a result, this current method seems to be a lot of more productive concerning the traditional form of antibacterial, decreases the use of antibiotics and associated reduces bacterial resistant. Nevertheless, the dynamics associated with the release of OTC from the ChNP-OTC structure are not yet clear and thus further studies are necessary.

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