# Nano-ceftriaxone as Antibiofilm Agent

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***Abstract***--- *Biofilms are communities of microorganisms that grow together and surrounded by an extracellular substance , which they form and prefer to adhere to living and non living surfaces. Metals like copper, gold, silver, titanium, and zinc are known to have antibacterial and antibiofilm properties, which present alternatives to antibiotics without increasing the risk of resistance. The current study introduce the nano-form of ceftriaxone as agent has the activity to decrease biofilm production of some virulent bacterial isolates.*

*Ninety bacterial isolates have been collected from different clinical sources. Identification was confirmed by API 20E and VITEK 2 systems. Antimicrobial susceptibility towards 16 antibiotics and minimum inhibitory concentration (MICs) of ceftriaxone before and after conversion were determined by using the two fold broth dilution method as a complementary test to verify the pattern of resistance.*

*MIC mean value of the nano-ceftriaxone was much less when compared with the MIC for micro-ceftriaxone at the same concentration. Bacterial isolates under study included 12 MDR isolates have been screened for their ability to produce biofilms with and without different concentrations of micro and nano-ceftriaxone ranges from (156- 10000)µg/ml. The results in general showed highly variation in the biofilm formation degree as well as the reduction percentage after treatment with different concentrations of micro and nano-ceftriaxone.*

***Keywords***--- *Biofilm, antibiotics, ceftriaxone, nano- ceftriaxone*

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## I. INTRODUCTION

The biofilm confers resistance to antibacterial agents through ; expression of chromosomally encoded resistant genes, limitation of antibacterial agents , decrease in growth rate and inactivation the host immunity (1 ) .In addition to increasing tolerance to molecules with antimicrobial activity, biofilms are related with chronic and persistent infections, they cause elevated costs to health care systems and patients every year because they are increase resistance to conventional antimicrobial drugs making the treatment not easy leading to high rates of morbidity and mortality (2 ).

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Eradicating a chronic infection associated with biofilm formation is so difficult since the bacterial biofilm is able to resist higher antibiotic concentration than bacterial suspension. Resistance to antibiotics may thus be seen as a phenotypic shift in behavior when cells adapt to a sessile lifestyle. Biofilm formation is regulated by expression of polysaccharide intracellular adhesion, which mediates cell to cell adhesion and is the gene product of icaADBC (3,4) .Cells in biofilms experience harsh growth environment. Survival depends on their ability to mutate and replace genetic information (5).

Nanoparticles of titanium dioxide have also established to inhibit biofilm formation(6). Use of titanium dioxide as an anti-bacterial agent has good potential as it is for applied previously in medicines and cosmetics. In addition to exhibiting anti-bacterial efficiency it is also promising to conjugate antibacterial agents onto the surface of titanium dioxide nanoparticles to assess the synergistic anti-bacterial action.The efficiency of the antibacterial agents that treating MRSA was increased in the presence of titanium dioxide nanoparticles. However, the action responsible for such improved anti-bacterial activity still needs to be studied (7). Silver impregnated hydroxyapatite and silver-titania matrices reduced bacterial adhesion and prevented biofilm generation by Gram-positive and Gram-negative bacteria, TiO2 acted as a better supporting matrix and prevented the aggregation of silver and allowed the controlled release of silver ions (8).
ZnO NPs have low toxicities in mammalian cells and more effective in inhibiting biofilm formation and growth of *E. faecalis*, *S. aureus*, *S. epidermidis*, *B. subtilis*, and *E. coli* than the NPs of other metal oxides (9).

## II. MATERIALS AND METHODS

**Bacterial isolates:** Ninety bacterial isolates have been collected from burns, wounds, ears swabs, urinary tract infections (UTIs), tracheal and sputum samples.

**Identification of bacterial isolates:** Morphological and biochemical tests (catalase, oxidase, motility, urease tests) have been used in bacterial identification . Results were confirmed by API 20E and VITEK 2 system**.**

**Antimicrobial susceptibility test**: All the bacterial isolates were tested for antimicrobial susceptibility towards 16 antibiotics according to the CLSI (2018) criteria, by using agar diffusion method .

**Preparation of nano- ceftriaxone**

The first step is preparation of a suspension of ceftriaxone which was chosen according to the results of antimicrobial susceptibility test.

The product was cooled, and the ice crystals of pure water formed at -18°C . The second step involves the sublimation of ice from the frozen product by passing heat air from shelf of lyophilization instrument to the frozen solution in the vial, the ice sublimes and the water vapor formed passes through the dried portion of the product to the surface of the sample, the water vapor is transferred from the surface of the product through the chamber to the condenser, and the water vapor condenses on the condenser. At the end of sublimation step, a porous plug is formed. Its pores correspond to the spaces that were occupied by ice crystals. The third step is drying which involved the removal of absorbed water from the product. All steps must be continuous about 48-72hrs. (11) using lyophilization instrument, LABCONCO Company (U.S.A) as shown in Figure (1) below:

**Figure (1)** Lyophilization instrument

**Minimum Inhibitory concentration (MICs ) test**

- Bacterial suspension of under study isolates was adjusted to the right A600 or Macfarland standard.
- The desired test antibiotic solution was prepared then diluted in Müeller Hinton broth to 2X the top concentration wanted in the test (e.g. if the highest test concentration is 256µg/mL, dilute to 512µg/mL).
- 100µL of Müeller Hinton broth was dispensed into all wells of the microtitre plate.
- 100µL of 2X antibiotic solution was pipetted into the wells of column 1.
- Using the pipette, the antibiotic was mixed by sucking up and down 5-8 times.
- 100µL from column 1 was removed and added to column 2. Mixed by sucking and transfer to column 3, the process was repeated to column 10.
- 100µL from column 10 was discarded.
- The bacterial inoculum was prepared to the size of $10^4$ to $10^5$ CFU/mL, by diluting it using broth or saline solution.
- Five µL of bacterial cells was poured into wells in columns 1 to 11. Bacteria was not added into column 12 (this will be the broth sterility control and blank for reading plates in a scanner)
- The plates were incubated in 37°C or other desired temperature for 12-18 hours.
- Experiments of MIC values were repeated in triplicate.
- The reading of results could be made manually using a black card or electronically with an ELISA reader (12).

**Effect of micro and nano-**ceftriaxone **in biofilm production (13 )**

❖ Bacterial suspension of twelve isolates under study were incubated on tryptic soy broth (containing 1% glucose) was added in microtiter plate in volume 100 µl in each well

❖ After 30 minutes 100 µl of antibiotic ( both forms ) were added to each well. The plate was incubated for 24hrs. / 37 °C.

❖ After incubation, all wells were washed with D.W to remove unattached cells.

❖ 200µl of 0.1% crystal violet was added to each well, shaking the plates three times to help the dye product to get the bottom of the well.

❖ After 15 minutes at room temperature, each well was washed with 200 µlD.W. This process was repeated three times. The crystal violet bound to the biofilm was extracted later with 200µl of ethyl alcohol.

❖ Absorbance was determined at 600nm in an ELISA reader.

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- Controls were performed with crystal violet binding to the wells exposed only to the culture medium as negative control and culture medium with bacteria as positive control.
- Experiments of biofilm values were repeated in triplicate.
- The reduction percentage in biofilm formation was measured using the formula (14):
- $\frac{\textbf{Control OD} - \textbf{Test Sample OD}}{\textbf{Control OD}} \times 100$ **Reduction =**

Optical density cut- off value (ODc) = average OD of negative control + 3x standard devation (SD) of negative control**.** Biofilm production can be divided into 3 classes as following:

| **Average OD value** | **Biofilm production** |
|---|---|
| ≤ OD /ODc < ~ ≤ 2x ODc | Non / weak |
| 2x ODc < ~ ≤ 4 x ODc | Moderate |
| > 4x ODc | Strong |

## III. RESULTS AND DISCUSSION

### Identification results

Bacterial isolates were preliminary cultured on MacConkey agar, blood agar, selective differential media and on CHROMagar Orient- ation medium in aerobic conditions followed by other differential diagnostic tests. Final identification for the isolates was dependent on the results of API 20E . By using this rapid and easy system, it was confirmed that twelve under study bacterial isolates were belonged to *E. coli, P.aeruginosa* , *K. pneumoniae* and *Enterobacter cloacae* .

Twenty –nine isolates were identified as *P. aeruginosa* (32.2%) while (27) isolates were *K. pneumoniae (30 %),* (12) isolates for*E. coli* (13.3%) *and* eleven isolates (12.2% ) were belonged to *each of S. aureusandE. cloacae* table(1):

**Table (1) Distribution of bacterial isolates according to their isolation sources**

| **specimen** | **No.** | ***P.aeruginosa*** | ***K.pneumoniae*** | ***S.aureus*** | ***E.cloacae*** | ***E. coli*** |
|---|---|---|---|---|---|---|
| Burn | 69 | 26 | 25 | 5 | 5 | **4** |
| Urine | 10 | 1 | | 4 | | **5** |
| Wound | 9 | 2 | 1 | | 6 | |
| Seminal fluid | 1 | - | | 1 | | |
| Sputum | 1 | | 1 | | | |
| Total No. | 90 | 29 | 27 | 11 | 11 | **12** |
| % | 100% | 32.2% | 30% | 12.2% | 12.2% | **13.3%** |

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Our results are in agreement with study of Shao *et al*., (15) who noticed the most common bacterial isolates in burn wound through ( 2009-2016) were *P.aeruginosa* which is followed by *K. pneumoniae* for Gram-negative bacteria, *S aureus* was the most common isolate for Gram-positive bacteria .

Identification results have shown the percentage of *K.pneumoniae* (30 %) and *S.aureus* (12.2%) which is consistent with study of Perween *et al* (16) that reported *Klebsiella* species was the second most frequent pathogen causing burn wound infection after *Pseudomonas* (28%), accounting for 22% of the total and 26% of all the Gram negative isolates. Our results were disagreed with study by Mundhada*etal* (17)which reported the isolation rate of Gram-negatives in burn infection was high and most common isolate was *K. pneumoniae* (34.40%) followed by *P. aeruginosa* (23.94%), *S.aureus* (22.94%), *E.coli* (7.34%) .

**Antimicrobial susceptibility test**

Antimicrobial susceptibility test was conducted for all under study isolates by using disc diffusion test to sixteen antibiotics of different classes includes ; Amoxicillin and Ampicillin, Cefotaxime, Ceftazidime, Ceftriaxon and Cefoxitin, Imipenem, Amikacin, Gentamicin, Ciprofloxacin, Levofloxacin, Norfloxacin, Nalidixic Acid, Aztreonam, Trimethoprim–sulfamethoxazole and Nitrofurantoin . Results were interpreted according to recommendation of CLSI, (10).

It was appeared that the resistance rates of all (16) antibiotics were as in figure 2; *P.aeruginosa*isolates (91.8 %), *K. pneumoniae* (70.2%), *S. aureus (70%), E. cloacae* (69 %) and (46.3 %) for *E. coli* .

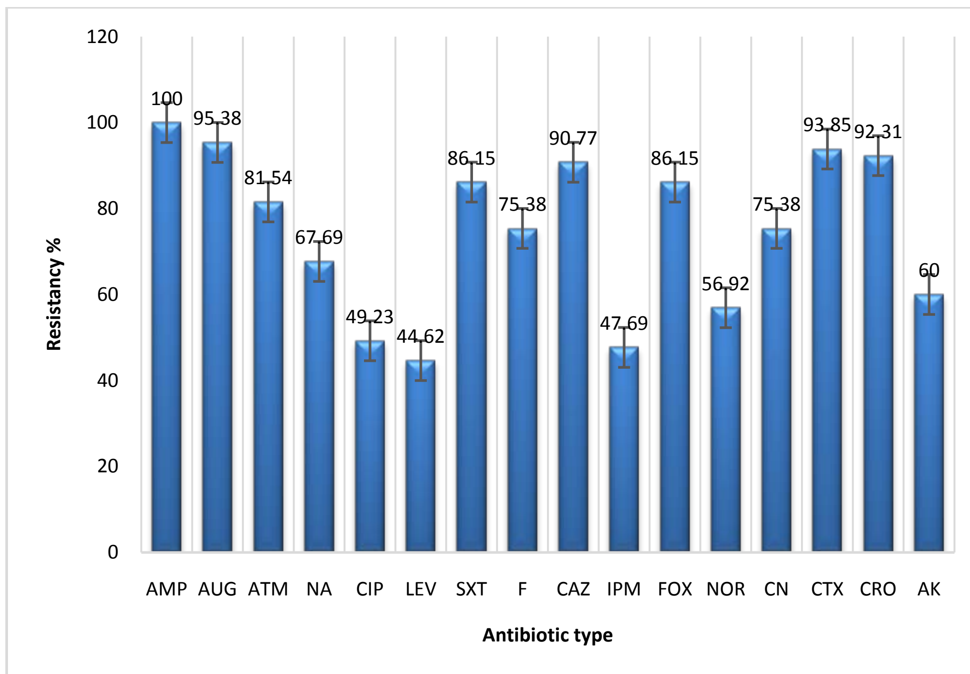


**Figure (2)** Resistance percentages for all bacterial isolates

*ampicillin(AMP),Amoxicillinclavulanate(AUG),Aztreonam(ATM),Nalidixicacid(NA),Ciprofloxacin(CIP),Levofloxacin(LEV),Trimethoprimsulfamethoxazole(SXT),Nitrofurantoin(F),Ceftazidime(CAZ),Imipenem(IPM),Cefoxitin(FOX),Norfloxacin(NOR), Gentamicin (CN), Cefotaxime(CTX), Ceftriaxone(CRO), Amikacin (AK)

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Figure (2) was showed the percentage of resistance for bacterial isolates involved in this study ; ampicillin( 100 %) , Trimethoprim–sulfamethoxazole (95.3%),Cefotaxime (93.8%) , Ceftriaxone (92.3%), Ceftazidime (90.7%),While Norfloxacin, Ciprofloxacin, Imipenem and Levofloxacin were (56.9 %),( 49.2 %), (47.6 %) ,(44.6 %) , respectively.

Antibiotic-resistant infections are now a serious global problem. In 2017, a strain of *K. pneumoniae* became resistant to all available antibiotics caused a fatal infection in the US (18). More than 0.7 million people die each year because of resistant infections and it was estimated that 10 million people will die from antimicrobial-resistant (AMR) infections in 2050. ESKAPE collection (*E.faecium, S.aureus*, *K.pneumoniae*, *Acinetobacterbaumannii, P. aeruginosa*, and *Enterobacter spp.*) was established that composed of critical and certain pathogens that easily acquire antibiotic resistance (19).

The current study revealed that all isolates includes were resistant to ampicillin, cefotaxime and ceftriaxon that what was compatible with study of Bhuiya *et al*., (20) which observed the antibiotic susceptibility patterns of Imipenem and meropenem were the most potent antibiotics followed by amikacin and piperacillin with maximum sensitivity. Gentamicin, ciprofloxacin, levofloxacin and aztreonam were found to be fairly active.

Here we would like to point out that ; from the ninety isolates were involved in this study, only (12) isolates were chosen that were resistant to most antibiotics used and have high resistant to ceftriaxone. According to this resistance, ceftriaxone was converted to nano-form to examine it's effect in biofilm formation. Yellowish powder of new particles of ceftriaxone was produced from conversion. The product was characterized by different spectroscopic and analytical techniques such as **UV-Vis spectral analysis** , **FESEM** ,**FTIR**, **XRD** and **AFM** which confirmed the formation of well dispersed new form of ceftriaxone .

**Minimum Inhibitory Concentrations (MICs)**

According to the identification results of VITEK 2 system and disc diffusion method, twelve bacterial isolates that belonged to the genera; *P.aeruginosa, K. pneumoniae, E.coli* and *S.aureus* were chosen to perform MIC test . These isolates were highly resistance for all antibiotics. MICs were determined by using the two fold broth dilution method as a complementary test to verify the pattern of resistance, A series of different concentrations ( 9.7 - 10000 µg/ml ) were prepared from both micro- and nano-ceftriaxone and tested as shown in table (2) which illustrated that results showedhighly significant p= 0.001 ($p \leq 0.05$)differences between the control and the all concentrations of ceftriaxone. The MIC results for ceftriaxone have shown that tested bacterial isolates at ceftriaxone concentration 10000 and 5000 µg/ml had the same significant effect were at mean (0.27) and (0.33) respectively compared with other concentration, in 2500 µg/ml, the mean was (0.40) it comes in the next p value level according to the LSD results while there was no significant difference between the remaining concentration ranges from (9.7-1250) µg/ml.

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**Table (2)** MIC of Ceftriaxone

| Concentration ug/ ml | Ceftriaxone | | | | Nano- Ceftriaxone | | | | *P value between the groups |
|---|---|---|---|---|---|---|---|---|---|
| | Minimum | Maximum | Mean± Std. Error | | Minimum | Maximum | Mean± Std. Error | | |
| Positive control | 0.64 | 0.99 | 0.78 | 0.03 | 0.64 | 0.99 | 0.78 | 0.03 | -------- |
| 10000 | 0.05 | 0.66 | 0.27a | 0.05 | 0.04 | 0.50 | 0.17a | 0.03 | 0.001 |
| 5000 | 0.04 | 0.77 | 0.33a | 0.06 | 0.03 | 0.45 | 0.19a | 0.03 | 0.001 |
| 2500 | 0.04 | 0.88 | 0.40b | 0.06 | 0.03 | 0.45 | 0.22b | 0.03 | 0.001 |
| 1250 | 0.21 | 0.79 | 0.52c | 0.04 | 0.04 | 0.52 | 0.27c | 0.03 | 0.001 |
| 625 | 0.24 | 0.85 | 0.55c | 0.03 | 0.07 | 0.45 | 0.31d | 0.82 | 0.001 |
| 312 | 0.31 | 0.78 | 0.60c | 0.04 | 0.16 | 0.56 | 0.33d | 0.02 | 0.001 |
| 156 | 0.30 | 0.97 | 0.67d | 0.04 | 0.19 | 0.57 | 0.39e | 0.02 | 0.001 |
| 78 | 0.53 | 0.92 | 0.72d | 0.03 | 0.18 | 0.56 | 0.38e | 0.02 | 0.001 |
| 39 | 0.58 | 0.99 | 0.74d | 0.03 | 0.25 | 0.58 | 0.40e | 0.02 | 0.001 |
| 19 | 0.50 | 0.87 | 0.77d | 0.04 | 0.18 | 0.59 | 0.39e | 0.02 | 0.001 |
| 9.7 | 0.54 | 0.69 | 0.71d | 0.05 | 0.21 | 0.53 | 0.38e | 0.03 | 0.001 |
| P Value Within the group | 0.001 | | | | 0.00 | | | | |

***ANOVA test was used to analyze repeated measure between tested concentration and control. Data expressed as mean±SE. LSD test was used to calculate the significant differences between tested mean, the letters (a, b, c, d and e) represented the levels of significant, highly significant start from the letter (a) and decreasing with the last one. Similar letters mean there are no significant differences between the tested mean.**

As for the MIC results of nano - ceftriaxone also the result showed highly significant p= 0.000 (p≤ 0.05)differences between the control and the all concentrations of nano- ceftriaxone. The MIC results for ceftriaxone have shown that tested bacterial isolates with nano- ceftriaxone at concentration 10000 and 5000 µg/ml had the same significant effect were at mean (0.17) and (0.19) respectively compared with other concentration , in 2500 µg/ml, the mean was (0.22) ,it comes in the next p value level according to the LSD results**.** The results of the statistical analysis showed a variation in the MIC by using the lowest subsequent concentrations for example the 625 and 312 µg/ml concentrations had the same MIC on tested bacteria , means

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were (0.31 , 0.33 ) respectively, while there was no significant difference between the remaining concentration ranges from (156– 9.7) µg/ml.

The current study was found that there were highly statistically significant in the MIC results between the ceftriaxone and the nano - ceftriaxonep= 0.001 (p≤ 0.05) at all of tested concentrations. The results clearly improved that the MIC mean value of the nano -ceftriaxone were much less when they were compared with the MIC for ceftriaxone at the same concentration.In addition the result found the highest concentration of ceftriaxone (5000 µg/ml) had the same MIC value for the much less of nano- ceftriaxone concentration at (312ug/ml) the MIC mean value were 0.33.

**Biofilm Formation degree and biofilm reduction percentage of Ceftriaxone**

The twelve bacterial isolates under study have been examined for their ability to produce biofilms with and without different concentrations of micro- and nano-ceftriaxone ranges from (156-10000)µg/ml. Biofilm Formation Degree (BFD) and Biofilm Reduction Percentage(%BR) were calculated as mentioned previously in materials and methods , the results were expressed by mean of optical density (O.D) ± SE. According to the current results, all tested bacterial isolates have ability to produce biofilm (100%) and classified as strong(S) biofilm producers.

When study the effect of MIC and sub MIC of micro and nano Ceftriaxone on BFD and %BR of the strong biofilm producer (bacterial isolates founded the following results, first the micro-CRO converts the strong degree biofilm producer isolates to the moderate degree at all tested concentrations , but with a different reduction percentage with significant differences ($P \leq 0.05$) , the highest %39.4 was at concentration 2500 µg/ml and 625 µg/ml , while the other concentrations came in the second rank percentage reduction %36.4 at (5000,312,156) µg/ml followed by the third rank %BR which was %33.4.Finally the lowest %BR (%30.3) was found with the highest concentration 10000ug/ml .

Secondly, the results show various percentages of reduction in biofilm formation when the strong biofilm producers bacteria treated with different concentration of nano-ceftriaxone , the current results have found this nano-biotic converts the strong degree biofilm producer isolates to the weak degree at the highest three concentration ranges from(2500-10000) µg/ml the %BR is %51.5 while the nano- ceftriaxone converts them from SBP to moderate biofilm producer at the lowest concentration ranges from 1250-156 µg/ml ,the %BR is equal to %48.48 at 1250 µg/ml and equal to %45.45 at all the remaining concentrations (625,312 and 156 µg/ml).

The results as well as noticed significant differences p=0.001 ($P \leq 0.05$) between micro(%30.3) and nano-ceftriaxone (%51.5) at 10000 µg/ml and p=0.01 ($P \leq 0.05$) in contrast with the other concentrations. And were found that less nano-CRO concentration (156 µg/ml) had a great biofim reduction percentage which was45.45% comparing with the highest %BR ;39.4% at 2500 µg/ml and 625 µg/ml. In addition , the nano-CRO found to be more effective on changing the biofilm formation degree and in the reduction of biofilm formation percentage in the lowest or highest concentration in contrast with micro-CRO had a greater effect on the lowest concentrations, but does not reach to the lowest %BR at the lowest concentration of nano-CRO (table 3) .

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**Table (3)** Biofilm production with different concentrations of nano-and micro- ceftriaxone

| Ceftriaxone Concentration ug/ ml | O.D of micro- ceftriaxone | | | | O.D of nano- ceftriaxone | | | | P value between the groups |
|---|---|---|---|---|---|---|---|---|---|
| | Mean± SE | | BFD | %BR | Mean± SE | | BFD | %BR | |
| Positive control | 0.33 | 0.01 | S | %100 | 0.33 | 0.01 | S | %100 | ------- |
| Negative control | .08 | .002 | ----- | ------ | .08 | .002 | ----- | ----- | ------ |
| 10000 | 0.23 | 0.03 | M | %30.3 | 0.16 | 0.01 | W | %51.5 | 0.001 |
| 5000 | 0.21 | 0.02 | M | %36.4 | 0.16 | 0.01 | W | %51.5 | 0.01 |
| 2500 | 0.20 | 0.03 | M | %39.4 | 0.16 | 0.01 | W | %51.5 | 0.01 |
| 1250 | 0.22 | 0.01 | M | %33.3 | 0.17 | 0.01 | M | %48.48 | 0.01 |
| 625 | 0.20 | 0.01 | M | %39.4 | 0.18 | 0.01 | M | %45.45 | 0.01 |
| 312 | 0.21 | 0.01 | M | %36.4 | 0.18 | 0.01 | M | %45.45 | 0.01 |
| 156 | 0.21 | 0.01 | M | %36.4 | 0.18 | 0.01 | M | %45.45 | 0.01 |
| P Value (+ve C vs tests) | 0.01 | | | | 0.001 | | | | |
| P Value Within the group | 0.05 | | | | 0.01 | | | | |

BF= Biofilm Formation Degree/%BR= Biofilm Reduction (Inhibition) percentage /Strong biofilm producers (<32)/M=Moderate (<0.16-≤32)/Weak (0.08-<0.16)/No biofilm≥0.08 (OD for negative control)

We couldn't compare the results concerned nano-ceftriaxone to other studies because we didn't find any identical results neither local or global, thus the current study had compared with the studies of loaded nanoparticles. Study of Kumar *etal*( 21) on ceftriaxone-loaded solid lipid nanoparticles (CL-SLNPs) use as nanocarriers to enhance drug delivery efficacy has been increasing in the healthcare field due to their tunable surface properties, antibacterial activity of the (CL-SLNPs) was evaluated against different Gram-positive and Gram-negative bacterial strains. The minimum inhibitory concentration of CL-SLNPs against *P. aeruginosa* was determined as 31 μg/mL

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# IV. CONCLUSION

Nano- ceftriaxone has superior activity to reduce the biofilm formation of certain pathogenic bacterial isolates.

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