Original Article

Cefotaxime Eluting Niosomes as A Novel Approach to Potentiate the Antibacterial Activity

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Abstract

Multidrug resistance has resulted in hurtful infections on treatment with common antimicrobials and is associated with health threatening problems. Antibiotic abuse in health care systems contributes to global resistance. β-lactams are the predominantly used antibiotics, and the most often used is cefotaxime, therefore facing a resistance problem. Potent cephalosporin derivatives, β-lactamase inhibitors, prodrugs, and drug nano-carriers are approaches to cutback bacterial resistance. An example of a nanocarrier applied in field of antibacterial activity enhancement is niosomes. Niosomes are self-assembled vesicles made from non-ionic surfactants with cholesterol also additives included or omitted that can provide good features such as antibiotic shielding, cleavage proofing, controlled release, and specific targeting. Cefotaxime niosomes preparation done by Thin-Film Hydration method using Tween 40, span 60, stearylamine, and cholesterol. The dried film was moisturised with buffered solution of cefotaxime at 45-55°C then the final nanosized niosomal cefotaxime was generated through a probe sonication. A 27 formulas were prepared and identified by dynamic light scattering so that polydispersity index and the vesicular size are measured. The selected formula of cefotaxime niosomes (CN6) has as showed the lowermost values and a prominent vesicular structure of 106 nm diameter on Transmission electron microscope and compatibility on Fourier Transform Infrared System analysis. CN6 remained unchanged in diameter and Poly-dispersity index after 100 days of storage at 2 to-80 C and also onsubsequent freeze drying and reconstitution. Entrapment efficiency was quantified by the ultracentrifugation method and was discovered to be 93 %. Antibiotic delivery by cefotaxime-eluting niosomes has been shown to be successful with antibacterial potentiation of 160, 8, 640 and 40 times better than aqueous solution against Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa respectively which is obviously a considerable drop in the minimum inhibitory concentrations of the selected formula versus the aqueous cefotaxime solution and this was evaluated using Agar well diffusion technique.

Keywords: Cefotaxime, Niosomes, Antibacterial activity potentiation, Nanotechnology.

1. Introduction

Bacterial resistance is a global challenge lead to high health problems. Multidrug resistance in gram-positive and gram-negative bacteria result in harmful infections on treatment with cephalosporins [1]. Therefore, necessary to develop novel treatment options and alternative antimicrobial therapies due to current lack of effective medications, ineffective preventative measures, and few number of new antibiotics in clinical pipeline.

Antibiotics are an essential medical treatments required for developing sophisticated medical procedure such as infectious disease and post-surgery management. Since their introduction into clinical practice in 1964, cephalosporins have placed a significant worldwide burden on bacteria's ability to develop resistance.

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The microbe's natural response to surviving is to fight back against its attackers. As a third-generation cephalosporin, Cefotaxime sodium targets both Gram positive and Negative bacteria, however it is more effective against Gram Negatives and has some effect on Pseudomonas. Its stable against the β-lactamase degradation [2]. Among the most productive strategies to improve β-lactams functions is to use nanoparticles, liposomes, and Niosomes. Nanocarriers may improve potency β-lactamases in long run by minimizing resistance to antimicrobials and assisting in delivering the medicine to the target sites. Niosomes are self-assembled vesicles made from synthetic surfactants with cholesterol with or without additives can acquire the desired form, size, and membrane properties by altering their content. Niosomes size, morphology, and exterior chemistry can alter the drug's intrinsic pharmacokinetics and medicine delivery. The niosomes have these unique characteristics from the goods of the surface. Nanocarriers as dosage forms can provide good features such as antibiotic shielding, cleavage proofing, controlled release, and specific targeting. Function as magic pills targeting diseased tissues, not healthy tissue. Nonionic surfactants self-assemble in aqueous mediums to form sealed bilayer structures (Figure 1) [3].

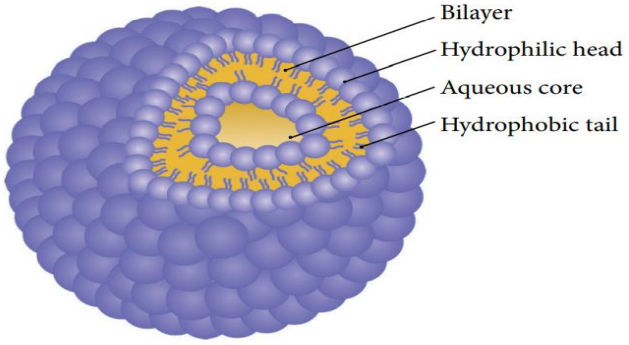


Figure 1: Assembly of Niosome, cited from [4].

2. Material and Methods

2.1 MATERIALS

Cefotaxime, Octadecylamine (stearylamine) and cholesterol were purchased from Baoji Gukang Bio Tech co, china. Span 60, tween 40, Phosphate buffer saline (PBS) and Chloroform were obtained from Thomas Baker, India. Muller-Hinton (MH) agar and Nutrient agar were purchased from oxoid company, Canada. All other reactants, solvents and media were of analytical grade or higher.

2.2 METHODS

2.2.1 Preparation of cefotaxime niosomes

When making cefotaxime niosomes (CN), the Thin-Film Hydration (TFH) method was used for 27 formulas. 2 ml Chloroform was used to dissolve surfactants (Tween-40 and Span-60), stearylamine (0.5 mg), and cholesterol. A rotary evaporator was used to extract the solvent at room temperature, a thin coating of solid content left on the inside wall. Using a water-bath agitator and 5 ml of cefotaxime sodium aqueous solution, the dried surfactant film may be rehydrated. Typical multilamellar niosomes were formed [5]. The dispersion is then probe sonicated (300Watts) for 3 minutes with 3 seconds on and 3 seconds off to produce tiny unilamellar niosomes [6-8]. The mixture placed in an ice-water bath to prevent overheating [9]. Each formula was filtered using 0.22 µm filter in order to minimize the size and polydispersity index (PDI) [10]. The prepared niosomes were dried using the freeze-drying technique to maintain good stability [11]. It's essential to establish that freeze-drying does not affect the size or characteristics of nanoparticles [2]. Table 1 shows the factorial design of experiments using three factors of drug loading, cholesterol: surfactants ratio, and Tween 40: Span 60 ratio, Showing different Cefotaxime sodium Niosomal formulations (CN).

Table 1: Different Cefotaxime sodium Niosomal formulations (CN).

Formula	Drug content (mg)	Cholesterol: Span60: Tween40 (molar ratio)
CN1	75	1:1:1
CN2	75	1:1:2
CN3	75	1:2:1
CN4	100	1:1:1
CN5	100	1:1:2
CN6	100	1:2:1
CN7	125	1:1:1
CN8	125	1:1:2
CN9	125	1:2:1
CN10	75	2:1:1
CN11	75	2:1:2
CN12	75	2:2:1
CN13	100	2:1:1
CN14	100	2:1:2
CN15	100	2:2:1
CN16	125	2:1:1
CN17	125	2:1:2
CN18	125	2:2:1
CN19	75	3:1:1
CN20	75	3:1:2
CN21	75	3:2:1
CN22	100	3:1:1
CN23	100	3:1:2
CN24	100	3:2:1
CN25	125	3:1:1
CN26	125	3:1:2
CN27	125	3:2:1

2.3 Characterization of Cefotaxime Niosomes

2.3.1 Niosomes size

Using the Zetasizer NanoZS® (Malvern-UK), sizes of CN had been determined using a dynamic scattering of light approach. Deionized water was used to dilute produced niosomes in a polystyrene cuvette, and the mixture was then subjected to laser light diffraction at a 173° angle. The results of transmission-electron microscopy are supported by the visualization of TEM [12].

2.3.2 Transmission Electron Microscope (TEM)

The morphology of hydrated noisome dispersions of cefotaxime sodium was examined by transmission electron microscope (TEM). In central laboratory department at college of pharmacy / Basrah University, Iraq. CN was diluted at 1:10 using distilled water, then a 5µL drop of the diluted CN6 was placed on a Formvar coated copper grid, and the extra amount was removed by 0.2 filter paper. A 2% uranyl acetate solution was dropped onto the grid. Within a minute, the unused staining solution was pressed through filter paper to remove any residue. Finally, grid was examined by STEM detector for ZEISS Field Emission Scanning Electron Microscope Supra 55VP [5].

2.3.3 Entrapment efficiency

Entrapment efficiency [13] of the CN6 was quantify using dialysis centrifugation [14] [13]. A dialysis bag with a molecular weight cutoff of 8000 14,000 Da was filled with 1 mL of CN6 and then the knots were knotted at both ends of the dialysis membrane then that was placed in a centrifuge tube with a capacity of 50 mL, and the centrifuge was spun for 45 minutes at 6000 rpm and 50 degrees Celsius. It was determined with the use of an ultraviolet spectrophotometer how much cefotaxime was contained in the tube [15]. The EE was estimated using the following equation:

$$\text{Entrapment efficiency \%} = \frac{\text{(Initial amount of drug – Free drug)}}{\text{Total amount of drug}} \times 100$$

2.3.4 Fourier Transforms Infra-Red Analysis.

It was determined that the samples included cefotaxime sodium, span 60, Tween 40, cholesterol, stearylamine, CN6 and physical mixture. Hydraulic presses capable of applying a force equal to five metric tons were used in the production of KBr discs. In an inert environment, the pellets were scanned at 4000–400 cm–1 using an FTIR instrument [13].

2.3.5 Stability of Cefotaxime Niosomes.

The stability test for CN6 was carried out using storage at two distinct temperatures.: (2-8) ° C and 37°C for 100 days and investigate the influence on the particle size as well as the PDI [16]

2.4 In Vitro Evaluation of the antibacterial Activity of Cefotaxime aqueous solution and Cefotaxime Niosomes

MIC test was applied to evaluate the antibacterial effect of tested agents (cefotaxime niosomes) against Gram positive bacteria (Staphylococcus aureus) and Gram negative bacteria (Escherichia coli, klebsiella pneumoniae and pseudomonas aeruginosa) [17].

2.4.1 Microbial Strains

Human-isolated bacterial strains are part of the collection housed in the microbiological laboratory of the College of Pharmacy, Al-Basrah University, Basrah, Iraq.

2.4.2 Antibacterial Tests

By using the agar well diffusion technique, the growth inhibitory impact was measured [18] [19]. Disposable loops were used to transfer bacteria to agar, which was then spread using cotton swaps then left for a full day at 37 degrees Celsius to allow the bacteria to become active. [20]. Following the activation of the bacteria, they were put in 3 ml of normal saline, and the turbidity of the system was calibrated using a spectrophotometer to a standard of 0.5 mcfarland. (1-2 108 CFU/ml) [21]. Then Mueller Hinton agar plates were swabbed with a suspension of each bacterial species using sterile cotton swab. At the beginning we prepare stock solution of 1000 microgram per each ml then test the four bacteria by 1000 µg, 200 µg, 40 µg and 20 µg. These four concentrations show nearly the same result for both cefotaxime sodium aqueous solution and cefotaxime niosomes. Therefore, we were preparing additional eight concentrations of lower magnitude (10, 5, 2.5, 1, 0.5, 0.25, 0.125 and 0.062 µg per ml) to examine the superiority of niosomal cefotaxime over cefotaxime aqueous solution.

The medium was punched with six millimeters diameter wells and filled with 50 µl of the test sample and let to diffuse for half hour at room temperature [18]. Measurement of the inhibitory zone diameter (IZD) was done using a ruler after the plates were maintained aerobically at 37° C for overnight. All tests were done in triplicate and the growth inhibitory effect was recorded. The inhibition zone was then used to calculate the MIC [19, 22].

2.5 STATISTICAL ANALYSIS

The One-Way ANOVA single factor was used for differentiation between the antibacterial activity of cefotaxime aqueous solution and the selected formula of cefotaxime niosomes.

3. RESULTS AND DISCUSSION

3.1 Size and Size Distribution

Nanocarriers may improve β-lactamase activity in the long run by minimizing resistance to antimicrobials and assisting in delivering the medicine to the target sites. Using these measures helps to improve antimicrobial action while also

reducing medication resistance difficulties [23]. On the surfaces of the colloidal particles, endogenous plasma proteins may be discovered. These proteins are scavenged by macrophage in the liver in addition to spleen and they are subsequently removed through the reticuloendothelial system. Endocytosis, which happens after the niosome contacts with the biomembranes and releases the encapsulated antibiotic, is the process that allows antibiotic to be supplied from niosomes into the cell. As a consequence of this, niosomes are excellent vehicles for the delivery of antibiotics [2].

The qualities of the niosomes, along with their functionality and potential to load, are influenced by the dimensions of the vesicle. The capacity of nanoparticles to destroy bacteria is related to their size. Smaller particle sizes are associated with higher antibacterial activity in a variety of distinct nanoparticle systems. The nanoparticles with an average diameters of 80 nm displayed the greatest efficacy in both suppressing MRSA development and killing these germs [24]. Therefore, determining the size of the vesicle is necessary for the evaluation of antibacterial activity. The degree of size homogeneity among vesicles may be evaluated using PDI, lower number indicates a greater degree of size uniformity [25]. The niosomes that have been created have a particle size distribution that shows a wide variety of sizes see Table 2.

Table 2: CN formulas size and PDI

Formula	Z-Average	PDI	Formula	Z-Average	PDI
CN1	116.6	0.172	CN15	133.5	0.129
CN2	170.3	0.11	CN16	189.8	0.242
CN3	113.8	0.15	CN17	174.9	0.243
CN4	120.2	0.181	CN18	128.9	0.151
CN5	150.4	0.119	CN19	173.4	0.209
CN6	106.6	0.128	CN20	199.5	0.186
CN7	138.3	0.3	CN21	144.1	0.179
CN8	147.2	0.091	CN22	181.2	0.242
CN9	122.4	0.17	CN23	180.2	0.265
CN10	308.3	0.444	CN24	144.4	0.181
CN11	166.3	0.175	CN25	161.5	0.215
CN12	142.8	0.261	CN26	210.2	0.153
CN13	201.4	0.135	CN27	154.6	0.204
CN14	176.4	0.108			

The CN formula particle size increases when increasing cholesterol quantity, but decreases when increasing the ratio of surfactant to cholesterol, as indicated in the table of formulae. In addition to that, it was abundantly evident that this observation was applicable to the polydispersity index, and the findings of Amir Mirzaiea confirmed these conclusions [5]. The particle size for the selected formula(CN6) was 106 nm see Figure 2. In general CN size getting lower value by energy application via probe sonication same as seen with rifampicine niosomes [26].

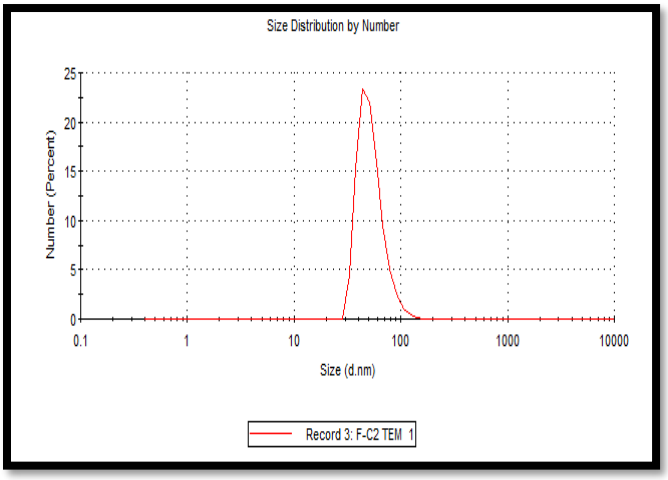


Figure 2: Particle size distribution by number of CN6.

3.2 Visualization by Transmission Electron Microscope(TEM).

The morphology of hydrated noisome dispersions of cefotaxime sodium (CN6) was examined by TEM. In central laboratory department at college of pharmacy / Basrah University, Iraq [5] see Figure 3.

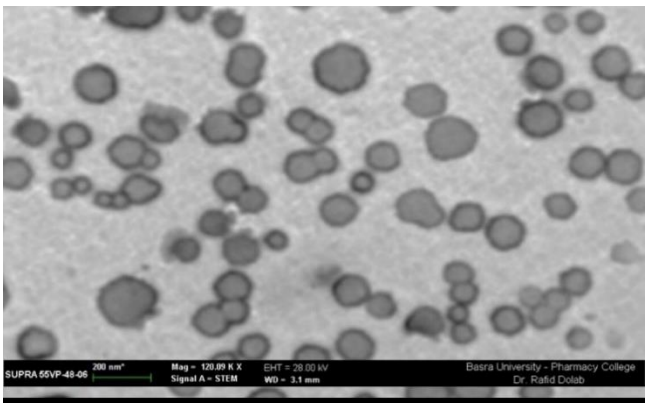


Figure 3: A TEM image of CN6 for morphological analysis.

The structure of niosomal cefotaxime was analyzed by TEM and is depicted in Figure 3. The picture proves that spherical niosomes were the ideal form. More so, the strict limits of the niosomes' structure were highlighted, verifying their spherical shape and smooth surface, as well as their consistent 100-135 nm size and lack of aggregation.

3.2.1 Fourier Transform Infra-Red

The FT-IR spectra of cefotaxime sodium powder (Figure 4) was compared with reference, the results reveal identical spectra for the tested powder and the reference [27]. Whereas the characteristic peaks of N-H symmetric stretching and O-H stretching are located at 3342 cm-1, the bands at 1759 and 1729 cm-1 are attributed to being caused by C=O stretching vibrations, and the peak at 2890 cm-1 is for CH3 symmetric stretching. Other important peaks can be found at 1546.96

cm-1 for the C=C stretching, 2922.25 cm-1 for the =C-H stretching, 1512.24 cm-1 for the N-H bending, 3387.11cm-1 for the strong and broad band of the O-H stretching, 1483.94 cm-1 and 1383.01 cm-1 respectively for the asymmetric and symmetric bending vibration of the CH3 group. The other functional groupings don't hold up very well. The ingredients used in the medicine's recipe did not have any negative interactions with the drug (CN6), and the formula was consequently allowed to undergo additional testing (Figure 5) [28]. The compounds span 60, tween 40, and cholesterol have numerous polar groups (C═O, OH, and NH3+) in each molecule that may be involved in intra H-bonding; thus, no chance for inter H- bonding with the drug. C-C stretching in the aromatic ring (1506 cm-1) and C=C stretching {1674 cm-1} peaks in cholesterol were not observed in FT-IR spectra of niosomes, providing further evidence for the trapping of cholesterol in the lipid bilayer and the development of niosomes [29].

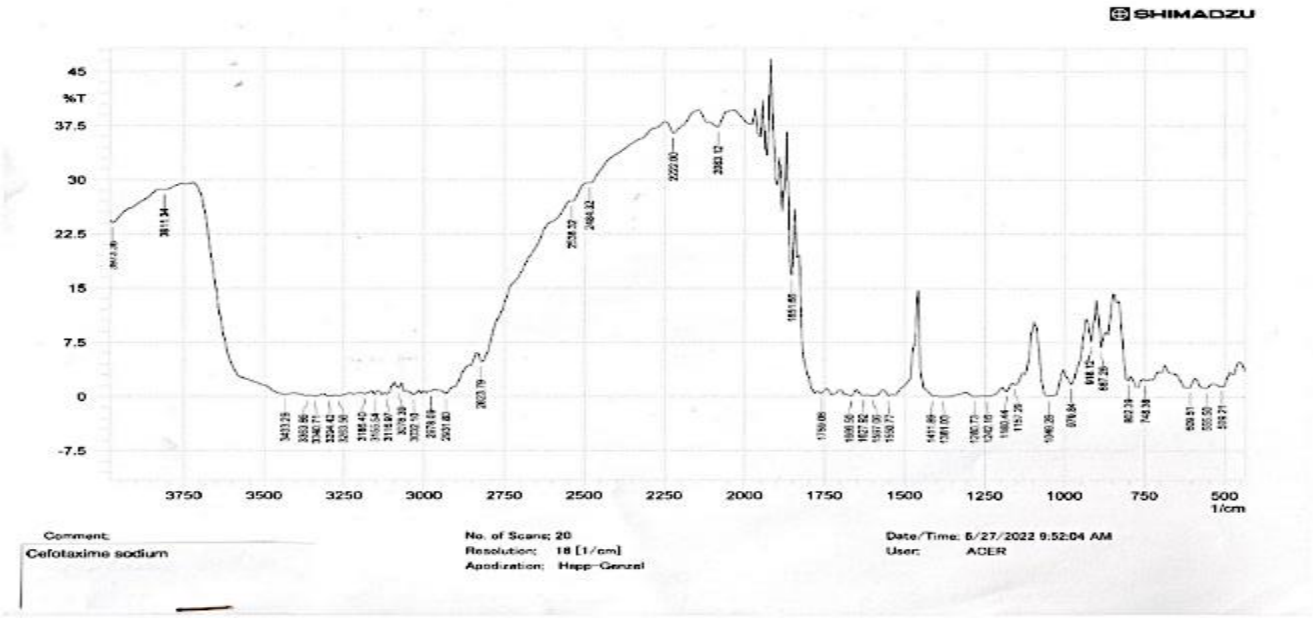


Figure 4: IR spectrum of Cefotaxime sodium

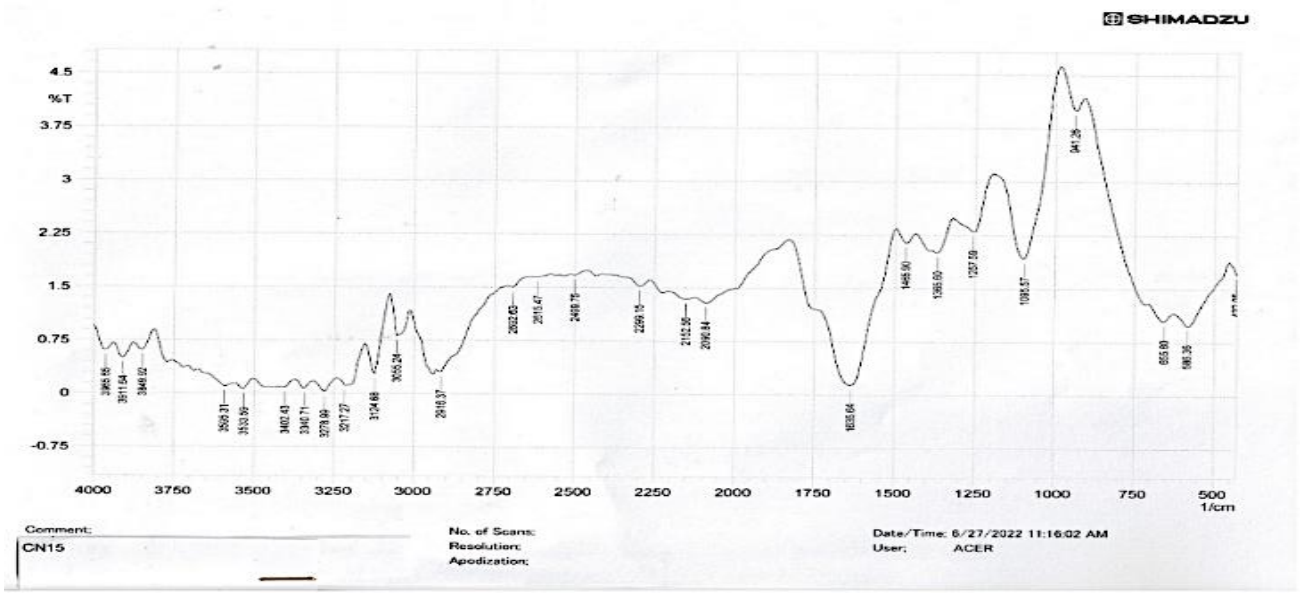


Figure 5: IR spectrum of CN6

3.3 Stability of CN6

A consistent particle size and PDI throughout time are necessary for stable niosomal dispersion. The membrane components, which are mostly insoluble in water, must not precipitate. Data on the stability of the chosen for CN6 showed that niosomal preparations kept in the refrigerator for up to 100 days showed no signs of color alteration see Table 3. The reasons for that are the molecular interaction of polar head groups of surfactants with the solvent and permeation of solvent into the bilayers [30]. Physical stability of CN6 was evaluated in terms PDI and particle size for 100 days at two

different temperatures. At temperatures ranging from 2 to 8o C, there was no discernible change in particle size [110. Therefore, Niosomes are so much more stable in the fridge than at 37°C as shown in Table 3 [16].

Table 3: Physical stability of the selected formula (CN6)

Day	Particle diameter (nm)	PDI	Particle size filtered	PDI filtered	Color
zero	134	0.18	126	0.10	Yellowish green
5	135	0.544	123	0.435	Yellowish green
15	130	0.444	111	0.323	Yellowish green
25	128	0.376	100.8	0.205	Yellowish green
45	140	0.543	102	0.232	Yellowish green
60	200	0.690	106	0.28	Yellowish green
75	164	0.638	124.1	0.368	Yellowish green
100	151	0.411	91	0.188	Yellowish green

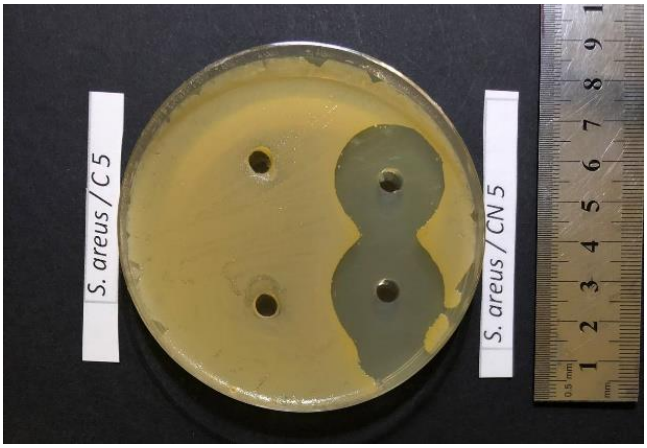
3.4 Entrapment efficiency(EE)

EE was determined to be the acceptable formulation. The percentage of cefotaxime sodium entrapped inside the niosomes was 86% for formulations that used the major medium being distilled water for hydration [5]. After buffering the hydration solution with PBS to a pH of 7.2, the amount of free cefotaxime that was not entrapped was reduced by up to 50 percent, which resulted in an entrapment efficiency of 93 percent which is goes with V ravalika [31]. This could be explained by fact that unionized drug entrapped more efficiently. This behavior might be explained due to the reality that span 60 exists in form of solid state at room temperature and exhibited greater phase transition temperatures [Tc] [32]. The same results were obtained from the encapsulation of gentamicin in niosome [33] and diclofenac diethyl ammonium [34].

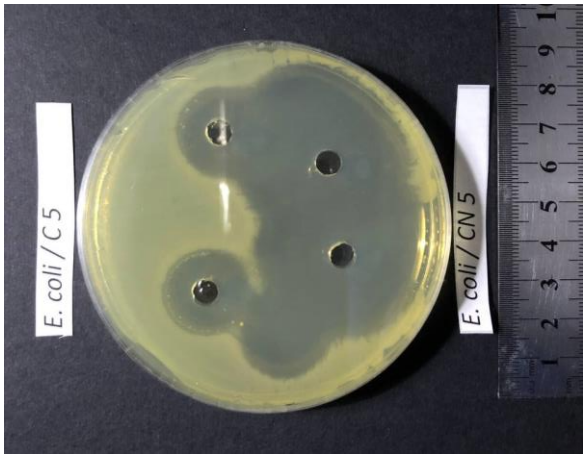
3.5 In Vitro Evaluation of the antibacterial Activity of Cefotaxime aqueous solution and Cefotaxime Niosomes

Strategies based on nanotechnology have become a more effective method for achieving localized significant antibacterial control of bacterial resistance and medical biofilms [35]. It has been shown that cationic liposomes adsorb more extensively onto biofilms of Staphylococcus aureus than anionic ones, according to various research on liposomes. Other researchers employed Pseudue aeruginosa as the primary biofilm developer and found that liposomally encapsulated ciprofloxacin reduced biofilm development on the catheter surface. If we compare our results with those from other tests, it is clear that our findings on the effects of adding stearylamine to niosomes are on the right track [36].

Niosomes are bilayer hydrated vesicles made of cholesterol and non-ionic surfactants, similar to liposomes. Niosomes alleviate the fundamental drawbacks of liposomes by being easier to handle, less costly, and more physicochemically stable. It is well knowledge that niosomes interact with phospholipid membranes and that this interaction both controls the release of antimicrobial medicines and their activity. When combined with lipophilic surfactants, hydrophilic surfactants, such as Tweens, may produce a hydrophilic bilayer. Additionally, it may reduce the attachment of germs to surfaces by enhancing hydrophilic medicament trapping abilities. The idea has been put out that antimicrobial-loaded niosomes might offer an alternative multifunctional technique for controlling bacterial susceptibility [37]. In vitro activity of niosome-encapsulated cefotaxime was evaluated by observing minimum inhibitory concentration solution against Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa (MIC) and compared to free cefotaxime (Figure 6).



A



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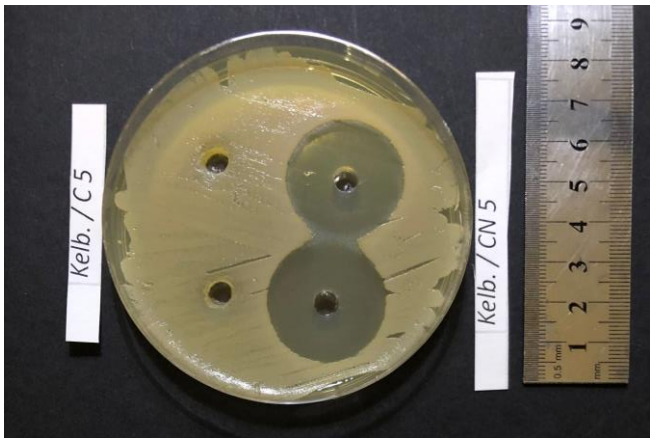

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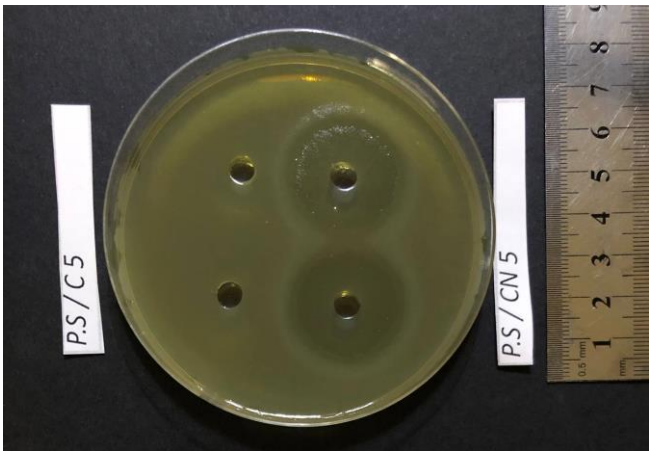

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Figure 6: Representation of Agar well diffusion MIC assay, where S.aureus/C5 is cefotaxime solution and and S.aureus/CN5 is cefotaxime niosomal selected formula (CN6) of 5 µg per each ml tested on (**A**) *Staphylococcus aureus,* (**B**) *Escherichia coli, (C) Klebsiella pneumoniae,* and (D) *Pseudomonas aeruginosa***.**

Below, the MICs have been calculated by determining the inhibition zone for CN6 and the free cefotaxime solution against Staphylococcus aureus, Escherichia coli, klebsiella pneumoniae and pseudomonas aeruginosa as shown in Table 4. The MIC for niosomal cefotaxime sodium versus solution of cefotaxime sodium determined, four different strains of bacteria were tested using the Agar well diffusion technique. When it comes to the effect of niosomal encapsulation on cefotaxime's MIC, positively charged niosomes (CN6) dramatically reduce it when compared to cefotaxime only. In the instance of CN6 for all test organisms, the reported a significant reduction in MIC ($P \leq 0.05$). For Staphylococcus aureus, klebsiella pneumoniae and pseudomonas aeruginosa high MIC values indicate a high degree of resistance in the free solution of cefotaxime., which is more than 20 µg/ml for these three strains. On other hands the MIC of free solution for Escherichia coli is 0.5 µg/ml. The MICs for aqueous cefotaxime sodium agreed with published values [38-42]. Incorporation of cefotaxime into niosomal vesicular system theoretically and practically reduce the MIC significantly.

Table 4: MIC for cefotaxime free solution and niosomal cefotaxime.

Bacteria	Reference cefotaxime MIC(/mL)	MIC for Aqueous cefotaxime solution (/mL)	MIC for niosomal cefotaxime(/mL)	Potentiation (folds)
Staph. aureus	0.5-2 µg	20 µg	0.125 µg	160
E. coli	4-16 µg	0.5 µg	0.062 µg	8
Kleb. pneumoniae	64 µg	40 µg	0.062 µg	640
Pseud. aeruginosa	8-512 µg	40 µg	1 µg	40

Our records show MICs values of 0. 0.125 µg/mL for Staphylococcus aureus, 0.062 µg/ml for Escherichia coli and klebsiella pneumoniae and 1 µg/ml for pseudomonas aeruginosa. A similar pattern was reported for liposomes containing Ciprofloxacin, meropenem and gentamicin [43, 44]. Cefotaxime-eluting niosomes, according to the research, might be an effective antibiotic delivery system Antibiotic delivery by cefotaxime-eluting niosomes has been shown to be successful with antibacterial potentiation of 160, 8, 640 and 40 times better than aqueous solution against Staphylococcus aureus, Escherichia coli, Klebsiella pneumoniae, and Pseudomonas aeruginosa respectively which is obviously a considerable drop in the minimum inhibitory concentrations.

It has been shown that cationic liposomes adsorb more extensively onto biofilms of Staphylococcus aureus than anionic ones, according to various research on liposomes. Other researchers employed Pseudue aeruginosa as the primary biofilm developer and found that liposomally encapsulated ciprofloxacin reduced biofilm development on the catheter surface. If we compare our results with those from other tests, it is clear that our findings on the effects of adding stearylamine to niosomes are on the right track [36].

4. Conclusions

A 27 formulas of Cefotaxime niosomes were prepared using Thin-Film Hydration method with tween 40, span 60, stearylamine and cholesterol and characterized using Zeta sizer to determine the vesicular size and the poly-dispersity index and CN6 was chosen as selected formula. Transmission-Electron Microscope results shows well defined vesicular structure of CN6 of 100 to 130 nm diameter with entrapment efficiency equal to 93 %. The niosomal antibacterial potentiation of cefotaxime evaluated by Agar well diffusion method and four strains of bacteria were used, Staphylococcus aureus, Escherichia coli, klebsiella pneumoniae and pseudomonas aeruginosa. CN6 shows great decline in MIC of cefotaxime compared to the free cefotaxime solution for all four strains of bacteria

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