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Antibacterial activity of clarithromycin loaded PLGA nanoparticles

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Novel drug delivery systems such as nanoparticles (NPs) have been proved to enhance the effectiveness of many drugs. Clarithromycin is a broad spectrum macrolide antibiotic, used in many infectious conditions like upper and lower respiratory tract infections, and skin and other soft tissue infections. This paper describes the preparation and enhanced *in vitro* antibacterial activities of clarithromycin loaded poly (lactic-co-glycolic acid) (PLGA) nanoparticles. A modified quasi-emulsion solvent diffusion (MQESD) method was used to prepare clarithromycin (CLR) NPs. The antibacterial activity of the NPs was evaluated using the agar well diffusion method against *Escherichia coli (PTCC 1330), Haemophilus influenzae (PTCC 1623), Salmonella typhi (PTCC 1609), Staphylococcus aureus* (PTCC 1112) and *Streptococcus pneumoniae (PTCC 1240*). The inhibition zone diameters related to each nano formulation were compared with those for untreated CLR at the same concentrations. The results indicated that the mean inhibition zone diameters of NPs against all the bacteria tested were significantly higher than those of untreated CLR, particularly in the case of *S. aureus*. The increased potency of CLR NPs may be related to some physicochemical properties of NPs like modified surface characteristics, lower drug degradation, and increased drug adsorption and uptake.

1. Introduction

There has been great progress in development of antimicrobial agents in the last century, but many infectious diseases, notably intracellular infections, are still difficult to treat. To some extent this difficulty is due to the complications related to the transport of antimicrobial agents through cell membranes and low activity inside the cells, as well as their toxicity to healthy tissues. Another major issue related to antimicrobials which significantly limits their use is the acquired resistance of infectious microbes. To address these issues, alternative antimicrobial drug delivery strategies have been proposed (Barrera et al. 2010).

Novel drug delivery systems offer numerous benefits over their conventional predecessors, including maximizing bioavailability, targeting the molecules, delivering drugs to the cell with precision and decreasing side effects, that have made them top research targets in the last few decades.

Nanoparticles, because of their small particle sizes and hence increased surface to volume ratio as well as quantum effects, exhibit modified physicochemical properties that enable them to cross biological barriers and transport their load into cells, while larger particles would have been cleared from the body (Hirn et al. 2011). The use of biodegradable materials to prepare nanoparticles allows for sustained drug release within the target site over a period of days or even weeks and thus increases the therapeutic benefit. These different properties make nanoparticles ideal drug delivery systems for the management of severe diseases such as intracellular infections or cancers, thereby overcoming some of the limitations of traditional therapeutics.

In fact, a number of nanoparticle-based antibiotics and anticancer delivery systems for infected or malignant cells, which utilise their superior physicochemical properties, have been approved for clinical uses and many other therapeutic nanoparticle formulations are currently under various stages of investigation (Heller 1980; Barrera et al. 1993; Jain 2000; Murakami et al. 2000; Diez and de Ilarduya 2006; Mohammadi et al. 2010).

It has been shown by Mohammadi et al. (2010) that azithromycin loaded PLGA nanoparticles in *in vitro* studies were more active than azithromycin solution against *Salmonella typhi*. The synthesis of vancomycin (Van)-capped Au nanoparticles and their enhanced *in vitro* antibacterial activities were recently reported. Also vancomycin-modified nanoparticles for efficient targeting and preconcentration of gram-positive and gram-negative bacteria have been developed and evaluated (Gu et al. 2008). In another investigation, the enhanced activity of ciprofloxacin nanoparticles in human macrophages infected with *Mycobacterium avium* has been reported in comparison with the free drug (Fawaz et al. 1998). Moreover Fattal et al. (1991) have demonstrated that ampicillin is more potent in the form of nanoparticles in murine salmonellosis. Clarithromycin is a broad spectrum antibiotic of the macrolide group, which is used in many infectious conditions such as upper and lower respiratory tract infections, and skin and other soft tissue infections, caused by various groups of bacteria such as *Escherichia coli, Haemophilus influenza, Staphylococcus aureus* and *Streptococcus pneumoniae* (PDR 2007).

Clarithromycin-loaded PLGA nanoparticles have been formulated by our team and their physicochemical properties have been studied, but the antimicrobial activity of the prepared nano formulations has not been investigated in detail in comparison with the unmodified drug. Therefore, the present study attempts to evaluate the antibacterial potencies of our clarithromycin-loaded PLGA nanoparticles against some strains

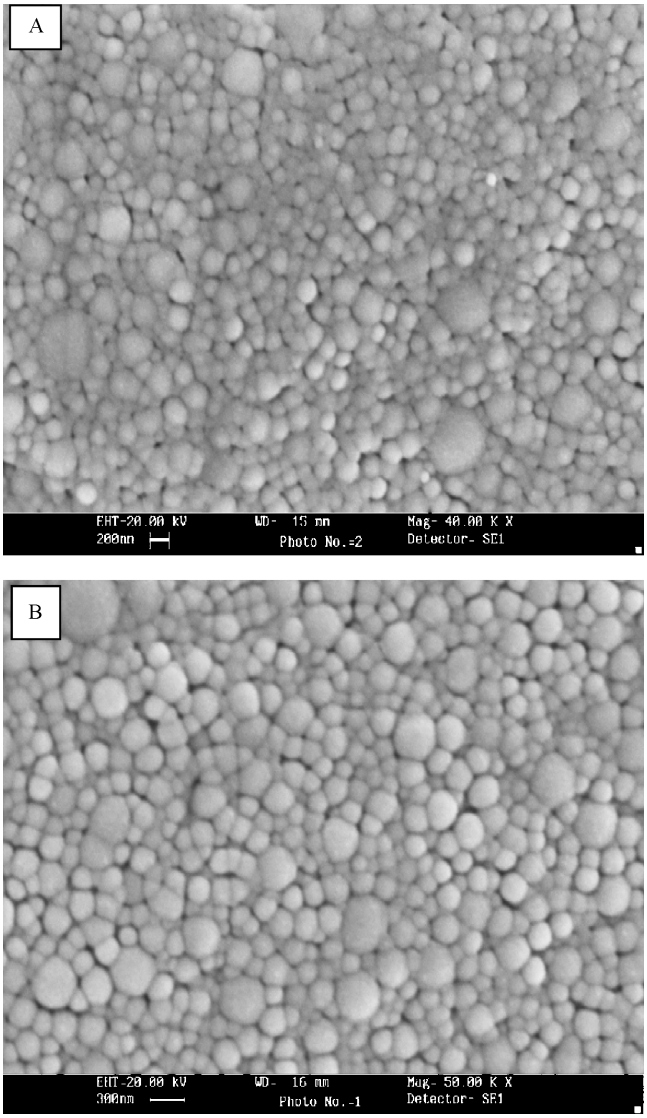

Fig. 1: SEM images of CLR loaded nanoparticles with (A) 1:1 (B) 1:2 drug to polymer ratio

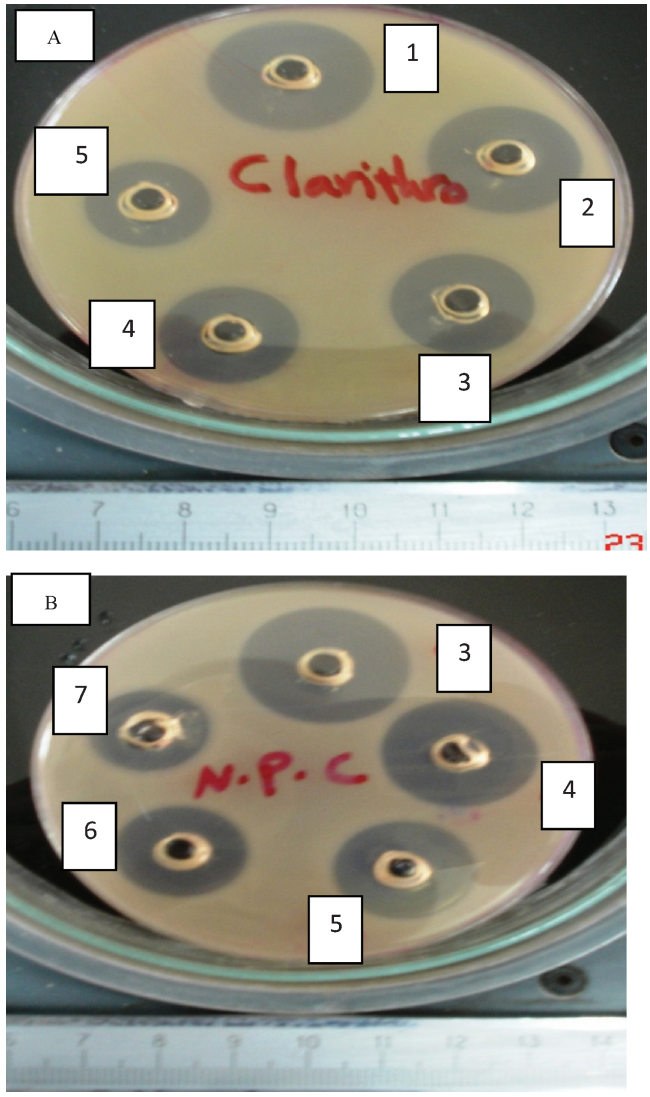

Fig. 2: Inhibition zone diameters of *S. aureus* in plates containing: (A) NANO1:1 (B) CLR solution of concentrations (μg/ml): 1 = 25.00, 2 = 12.50, 3 = 6.25, 4 = 3.12, 5 = 1.56, 6 = 0.78, 7 = 0.38

in its antimicrobial range including gram positive strains of *Staphylococcus aureus* and *Streptococcus pneumoniae* as well as the gram negative bacteria *Escherichia coli, Haemophilus influenzae, Salmonella typhi,* and to compare the results with pure clarithromycin.

2. Investigations and results

2.1. Physicochemical properties of CLR loaded nanoparticles

Submicron particles with a narrow size distribution ranging from 189 to 280 nm, a relatively monodisperse distribution and a spherical shape (Fig. 1) were prepared using a modified quasi-emulsion solvent diffusion technique. At pH 8, the ζ-potential of the intact polymer and CLR were -21.42 ± 2.84 and 1.47 ± 1.61 mV respectively. Because of the neutral charge of CLR at pH 8, on increasing the amount of PLGA in the nanoparticles, their surface charge became more negative. The ζ-potential of nanoparticles with a ratio of CLR to PLGA of 1:1 to1:3 ranged from -6.3 ± 1.70 to -14.26 ± 1.92.

The encapsulation efficiency of CLR loaded nanoparticles was between 57.4 and 80.2%, and maximum encapsulation efficiencies were achieved by increasing the PLGA to CLR ratio. CLR has at least three crystalline forms (Form I, Form II, Form O), form II being the more stable polymorph. Our results indicated that dominant XRPD peaks for intact CLR are comparable to those that reported for crystalline form II (Sohn 2000). XRPD patterns of NPs were characterized by the complete absence of any diffraction peaks, suggesting a complete amorphization of CLR in the NPs. Based on the results of the release study, the release of CLR from NPs was slower and more sustained than that of intact CLR, which may be due to the presence of insoluble polymer (PLGA) in the NP matrix, which in turn reduces water penetration, and hence dissolution and diffusion (Mohammadi et al. 2010).

2.2. Antimicrobial activity of CLR loaded nanoparticles

The agar diffusion method was used to evaluate the antibacterial activities of the selected nano formulations and untreated CLR samples against the indicated bacteria based on NCCLS guideline with some modifications (NCCLS 1996). Mean inhibitory zone diameters with each of the nano formulation of CLR were compared with their untreated counterparts at the same concentrations against the test bacteria using ANOVA SPSS 17. Figs. 2 and 3 show the inhibition zone diameters of the 1:1 and 1:2 nano formulations on plates containing *S. aureus* and *S. pneumoniae* in comparison with untreated CLR.

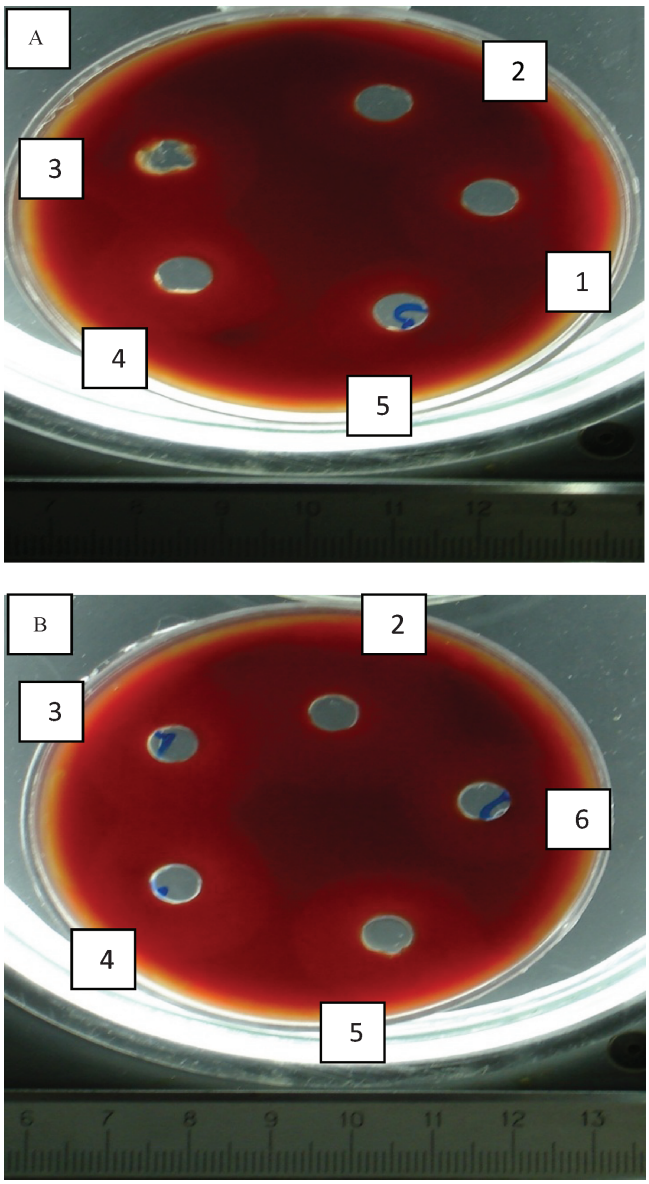

Fig. 3: Inhibition zone diameter of *S. pneumoniae* in Blood Base Agar medium: (A) NANO1:2 (B) CLR solutions at concentrations of (μg/ml): 1 = 12.50, 2 = 6.25, 3 = 3.12, 4 = 1.56, 5 = 0.78, 6 = 0.39

Figs. 4–8 illustrate and compare the mean inhibitory zone diameters for CLR loaded nonoparticles with those for untreated CLR against *E. coli, H. influenzae, S. typhi, S. aureus* and *S. pneumoniae* respectively. As it can be seen from Fig. 4, the mean

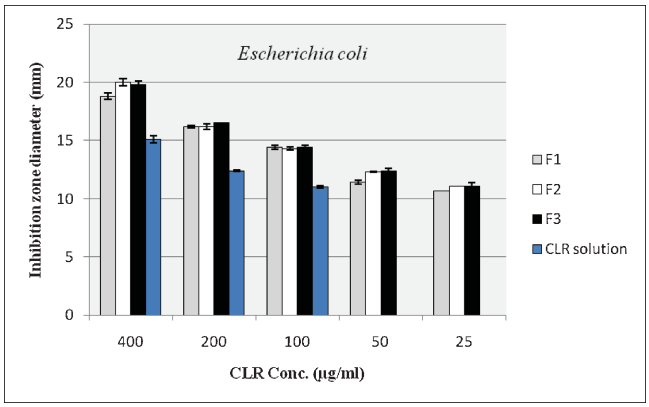

Fig. 4: Inhibition zone diameter CLR loaded nanoparticles against *E. coli* (NANO 1:1 (F1), NANO 1:2 (F2), NANO 1:3 (F3))

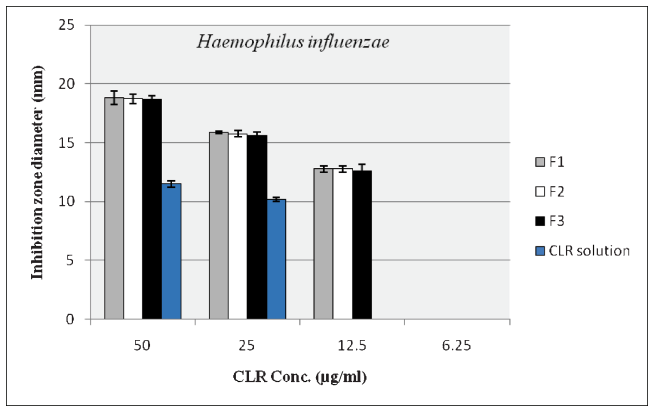

Fig. 5: Inhibition zone diameter of CLR loaded nanoparticles against *H. influenzae* (NANO 1:1 (F1), NANO 1:2 (F2), NANO 1:3 (F3))

inhibitory zone diameters against *E. coli* using nanoformulations are remarkably bigger than those with untreated CLR at the same drug concentrations. For instance, a mean inhibitory zone diameter against *E. coli* of about 12 mm was produced using a concentration of 100 μg/ml of untreated CLR, whereas prepared CLR loaded nanoparticles showed the same mean inhibitory zone diameter (12 mm) at a concentration of 25 μg/ml. As a result, it can be concluded that the antibacterial activity of CLR loaded nanoparticles increased 4-fold in the case of *E. coli*.

Also, Fig. 5 shows a significant increase in mean inhibitory zone diameter of CLR loaded nanoparticles compared to untreated CLR against *H. influenzae*, as untreated CLR at 12.5 μg/ml did not inhibit the growth of the bacterium (no inhibition zone), whereas CLR loaded nanoparticles at that concentration produced an inhibition zone more than 12 mm in diameter.

In the same way, the antibacterial activity of CLR loaded nanoparticles was enhanced against *S. typhi, S. aureus and S. pneumoniae* compared with untreated CLR as shown in Figs. 6–8. With untreated CLR, the first inhibition zone appears at concentrations of 100 μg/ml, 0.78 μg/ml and 0.19 μg/ml against *S. typhi, S. aureus and S. pneumoniae* respectively, whereas the concentrations decline to 50 μg/ml, 0.1 μg/ml and 0.1 μg/ml with CLR nanoparticles, indicating a 2-fold reduction of MIC values for CLR in nanoformulations against *S. typhi and S. pneumoniae,* and an 8-fold reduction in the concentration of CLR required to produce the same action against *S. aureus*.

Furthermore unloaded nanoparticles of PLGA showed no antibacterial activity against the indicated bacteria. Also the ratio of polymer used had no influence on the antibacterial activity of the nanoparticles, at least for the indicator bacteria examined here ($P > 0.01$). According to our study, the increase in the

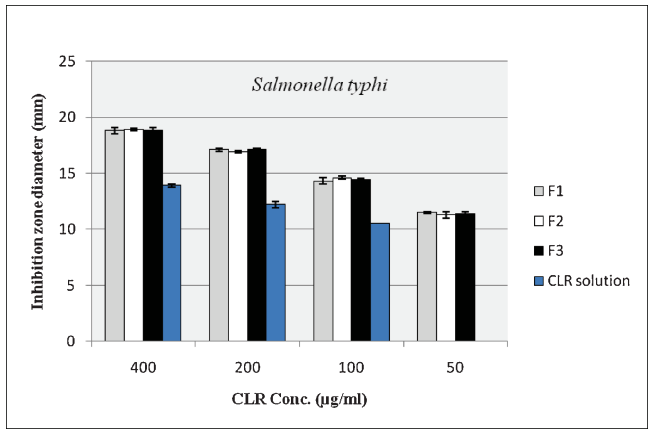

Fig. 6: Inhibition zone diameter of CLR loaded nanoparticles against *S. typhi* (NANO 1:1 (F1), NANO 1:2 (F2), NANO 1:3 (F3))

efficiency of CLR in the nanoformulation prepared is greater against the gram positive strain, *S. aureus,* than against the gram negative species tested, *E. coli, H. influenzae* and *S. typhi*.

3. Discussion

Nanotechnology has recently gained a significant role in different aspects of pharmaceutical sciences, especially in the development and optimization of novel drug delivery systems. A nanoparticle refers to a microscopic particle with at least one dimension less than 100 nm. On this scale, materials have unique physicochemical properties including ultra small size, large surface to mass ratio, high reactivity and unique interactions with biological systems. Drugs in nanoformulations exhibit improved pharmacokinetics, therapeutic index and serum solubility. Other benefits of these formulations include a longer systemic circulation life, sustained and controlled release of the drug, and targeted delivery of drugs to tissues and cells (Heller 1980; Jain 2000; Murakami et al. 2000). Moreover, drug-loaded nanoparticles can enter host cells through endocytosis and then release their drug payloads to treat microbial intracellular infections (Barrera et al. 1993; Zhang et al. 2010).

According to our results the antibacterial efficacy of CLR loaded nanoformulations was significantly increased in comparison with untreated CLR at the same concentrations against our tested bacterial strains of *S. aureus* and *S. pneumonia, E. coli, H. influenzae,* and *S. typhi*. The agar well diffusion method, a highly recommended method to evaluate the antimicrobial efficiency of antimicrobial agents, was used. The highest increase in the inhibition zone diameter of CLR loaded nanoformulations was observed against gram positive *S. aureus,* with an 8-fold reduction in the CLR concentration required to produce the same action, compared to untrated CLR. Enhanced activity of nanoformulations against *E. coli, H. influenza, S. pneumonia* and *S. typhi* was also found. This improved efficacy against microorganisms may be due to a number of mechanisms resulting from the unique physicochemical properties of nano scale particles, which can be classified into two main areas: (a) fusion of nanoparticles with the microbial cell wall or membrane and release of the loading within the cell wall or membrane; (b) adsorption of nanoparticles to the cell wall, thus serving as a reservoir for continuous release of drug molecules, which will diffuse into the interior of the microorganism (Barrera et al. 1993; Esmaeili et al. 2007; Zhang et al. 2010). Also the greater stability of the drug in nano encapsulated form against rapid enzymatic/hydrolytic degradation is an important factor (Panyam and Labhasetwar 2003).

Whether resulting from one mechanism or a combination of them, it is important to note that preparing CLR in the form of nanoparticles not only improves the physicochemical characteristics of the drug, but also maintains and even improves its antibacterial activity.

This study showed that PLGA could be a useful nanocarrier for CLR. There are numerous studies that report that antibiotic-loaded nano formulations have better antibacterial effects than the free antibiotic (Henry-Michelland et al. 1987; Cavallaro et al. 1994; Pinto-Alphandary et al. 2000; Dillen et al. 2004; Turos et al. 2007 a,b; Abeylath et al. 2008; Dillen et al. 2008; Jeong et al. 2008; Cheow and Hadinoto 2010). The modified surface characteristics of nanoparticles enable them to adhere to or merge with the bacterial cell wall and then release their drug content. These events could provide sustained antimicrobial activity of the drug incorporated against the target organism (Dillen et al. 2008). As a result, the efficacy of nano formulations can be improved.

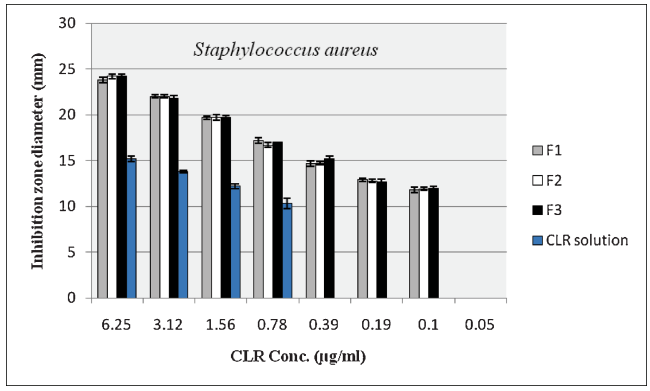

Fig. 7: Inhibition zone diameter of CLR loaded nanoparticles against *S. aureus* (NANO 1:1 (F1), NANO 1:2 (F2), NANO 1:3 (F3))

In conclusion, CLR-loaded poly (lactide-co-glycolide) (PLGA) nanoparticles were more effective than untreated CLR against *E. coli*, *H. influenza*e, *S. aureus* and *S. pneumoniae* and can be used to formulate new dosage forms. Further studies are required to evaluate the efficiency of the prepared nanoformulations in animal models as well as in healthy volunteers.

4. Experimental

4.1. Materials

Clarithromycin (CLR) powder was purchased from Elder Pharmaceutical Company (India). Poly (D,L-lactide-co-glycolide) (PLGA) (50:50 D,L-lactide:glycolide) with average molecular weight of 12000 g/mol (Resomer RG 502), was obtained from Boehringer Ingelheim (Germany). *Escherichia coli* (PTCC 1330), *Haemophilus influenzae* (PTCC 1623), *Salmonella typhi* (PTCC 1609), *Staphylococcus aureus* (PTCC 1112) and *Streptococcus pneumoniae* (PTCC 1240) were purchased from the Persian Type Culture Collection (Iran). Poly vinyl alcohol, PVA, with a molecular weight of MW 95000 from Acros Organics (Geel, Belgium) and acetone from Merck (Darmstadt, Germany) were used. Haemophilus Selective Agar, SoyBean Casein Digest Agar and Antibiotic assay Medium were purchased from Merck, Germany. Blood Base Agar medium was purchased from DIFCO, U.K. All other materials used were of analytical or HPLC grade.

4.2. Methods

4.2.1. Preparation of CLR loaded nanoparticles

Nanospheres were prepared according to our previously published method (Mohammadi et al. 2010). Briefly NPs (nanospheres) with 1:3, 1:2 and 1:1 ratios of drug to PLGA were prepared by nano-precipitation according to the modified quasi-emulsion solvent diffusion technique. CLR and PLGA powders were co-dissolved in an internal phase containing acetone, 2.5 ml, at room temperature (25 °C). Typically, different ratios of drug and polymer, to a total amount of 100 mg, were co-dissolved in acetone. The resulting organic solution was injected at a constant rate of 0.5 ml/min into

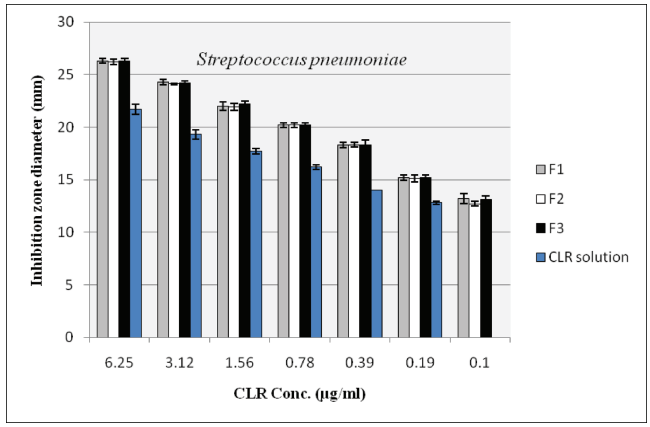

Fig. 8: Inhibition zone diameter of CLR loaded nanoparticles against *S.pneumoniae* (NANO 1:1 (F1), NANO 1:2 (F2), NANO 1:3 (F3))

an aqueous phase (40 ml) containing PVA 95000 (2% w/v), as a stabilizing agent. As the solubility of CLR in water decreases at higher pHs (Erah et al. 1997), the pH of the aqueous phase was adjusted to 8 in order to increase drug loading. The process was carried out under homogenization for 5 min using a Silent Crusher M (Heidolph, Germany) with an agitation speed of 13000 rpm in an ice-water bath. The organic phase was eliminated at room temperature under stirring for 12 h. The final nano-suspension was centrifuged (Beckman Centrifuge, Avanti^{TM} J-25, USA) at 14000 rpm for 30 min and the precipitated NPs were washed twice with water, using the previously described centrifugation approach, and then lyophilized with a lyophilizer (Christ Alpha 1–4; Germany). The final dry powder was taken for anti-bacterial investigations.

4.2.2. *Physicochemical properties of CLR loaded nanoparticles*

The mean particle-size of NPs were measured using a laser diffraction particle-size analyzer (Sald 2101, Shimadzu, Japan) equipped with Wing software (version 1.20) (Mohammadi et al. 2010). The morphology of the NPs was investigated by scanning electron microscopy (SEM) (LEO 440i, Leo Electron Microscopy Ltd, Cambridge, UK) at an accelerating voltage of 20 kV (Mohammadi et al. 2010). The particle surface charge was quantified as zeta potential (ζ potential) using a Zetasizer 4 (Malvern Instruments, UK). Measurements were performed in distilled water adjusted with sodium hydroxide to pH 8 and a conductivity of 50 μS/cm. In order to determine the encapsulation efficiency of the NPs, three samples were dissolved in acetone, and after evaporation of the acetone the drug was dissolved in 100 ml sterile water. The mixture was then centrifuged at 14000 rpm and the supernatant was drawn off to measure the CLR content by a fully validated HPLC method (Zakeri-Milani et al. 2009). X-ray powder diffraction (XRPD) patterns of the drug, PLGA, NPs and a physical mixture with the ratio of drug to polymer mentioned above were recorded using an automated X-ray diffractometer (Siemens D5000, Munich, Germany) (Mohammadi et al. 2010). Drug release was performed in sink conditions according to a previous publication for piroxicam NPs with some modification (Valizadeh et al. 2007).

4.2.3. *Nanosuspension preparation*

Suspensions containing 0.5 mg/ml of CLR were formulated by dispersing CLR powder alone, CLR-PLGA nanoparticles at the ratios of 1:3, 1:2 and 1:1, their corresponding physical mixtures, and PLGA nanoparticles without drug in 0.9% NaCl containing 0.005% w/w HPMC K100LV as a dispersing and viscosity adjusting agent. The pH of the final nano-formulations was adjusted to 7.4.

4.2.4. *Antibacterial activity of nanoparticle suspensions*

The antimicrobial activities of the prepared nanoformulations were compared with those of untreated CLR by the agar well diffusion method as well as by Minimum Inhibitory Concentration determination against *Escherichia coli, Haemophilus influenzae, Salmonella typhi, Staphylococcus aureus* and *Streptococcus pneumoniae* (NCCLS 2000). The bacteria, obtained in lyophilized form (Pasteur Institute, Iran), were initially suspended in sterile distilled water then activated according to the manufacturer's protocol. Recommended media for activation of *E. coli*, *H. influenzae*, *S. aureus* and *S. pneumonia*e were Soy Bean Casein Digest Agar, Haemophilus Selective Agar, Nutrient Broth and Blood Base Agar medium respectively. Suspensions of the activated bacteria in 0.9 % saline solution (30% turbidity at 580 nm) were used as working inoculums. Also stock samples of activated bacteria containing 20% glycerol were prepared and kept frozen for further use.

To perform the agar well diffusion assay, an appropriate amount of prepared inocolum for each bacterium was transferred into its selected medium. Wells of 8-mm diameter were punched into the surface of the agar medium using a sterile cork borer. Aliquots of 100 μl of each test compound solution (untreated CLR, nanoparticles with a 1:3, 1:2 and 1:1 ratio of CLR to PLGA, their corresponding physical mixtures, and PLGA nanoparticles without drug) were transferred into the wells. After incubation at 37 °C for 24 h, the inhibition zones around the wells were measured in millimeters (NCCLS 2000). All experiments were performed on three separate occasions and results were reported as mean ± SD.

4.2.5. *Statistical analysis*

One-way analyses of variance (ANOVA) were performed for comparison of the results. P values of $0 < 0.01$ were considered significant.

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