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Azithromycin-Loaded Solid Lipid Nanoparticles as a nanodrug delivery Strategy against Multidrug-Resistant *Escherichia coli*: Preparation, Characterization, and *In-vitro* Evaluation

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Abstract: The global rise of multidrug-resistant bacteria has intensified the demand for new antimicrobial agents and advanced delivery systems. This study aimed to evaluate the synergistic antibacterial activity and underlying mechanisms of azithromycin (AZM)-loaded solid lipid nanoparticles (SLNPs) against *Escherichia coli*. SLNPs were synthesized *via* the hot homogenization technique and subsequently encapsulated and loaded with AZM to assess release kinetics and antibacterial efficacy. Physicochemical characterization of both blank and AZM-loaded SLNPs was conducted using transmission electron microscopy (TEM), Fourier-transform infrared spectroscopy (FTIR), zeta potential analysis, and *in-vitro* antibacterial assays. The results revealed that the SLNPs exhibited a spherical morphology, and their particle size increased by approximately 61.26% upon AZM loading. *In-vitro* release studies demonstrated a sustained and controlled drug release profile, reaching 37.2% over 12 hours. Importantly, AZM-loaded SLNPs at 50 mg ml^{-1} exhibited a 9.01% higher antibacterial activity against *E. coli* compared to conventional AZM formulations, indicating improved efficacy and potential for dose reduction. These findings highlight the promising role of SLNPs as a nanocarrier platform to enhance the therapeutic performance of azithromycin in combating *E. coli* infections. In conclusion, solid lipid nanoparticles serve as an effective nanodrug delivery system to improve the antibacterial action of azithromycin in a dose-dependent manner, offering a potential strategy to combat antibiotic resistance in Gram-negative bacteria such as *E. coli*.

Keywords: Azithromycin, drug delivery, *E. coli*, solid lipid nanoparticles.

Abbreviations: AZM; Azithromycin, MIC; minimal inhibitory concentration, SLNPs; solid lipid nanoparticles, TEM; transmission electron microscopy.

1.Introduction

In recent years, increasing attention has been directed toward the application of nanotechnology in pharmaceutical delivery. Nanoscale drug carriers, with particle sizes ranging from 10 to 1000 nm, offer advantages such as improved drug absorption, targeted delivery, and reduced systemic toxicity [5,6]. These nanocarriers, whether composed of natural or synthetic, biodegradable or non-biodegradable materials, can encapsulate the therapeutic agents within their matrix or adsorb them onto their surface [7–9]. Such strategies enhance bioavailability and minimize undesirable side effects, contributing to better treatment efficacy [10].

Among the available nanocarrier systems, lipid-based nanoparticles particularly solid lipid nanoparticles (SLNPs) have garnered substantial interest due to their favorable physicochemical and biological properties [11,12]. SLNPs are colloidal particles with diameters typically ranging from 50 to 1000 nm and are composed of biocompatible, biodegradable lipids that remain solid at both room and physiological temperatures [13]. These nanocarriers combine the benefits of

polymeric nanoparticles and liposomes, offering high drug-loading capacity for both hydrophilic and lipophilic drugs, and demonstrate promising antibacterial performance against both planktonic and biofilm-forming pathogens [14–16]. Moreover, SLNPs exhibit sustained-release behavior and increased surface area-to-volume ratios, which contribute to enhanced drug efficacy, reduced dosing frequency, and improved therapeutic value [12].

Escherichia coli is a rod-shaped, Gram-ve, facultative anaerobic bacterium belonging to the genus Escherichia. Although many strains are commensal inhabitants of the intestinal tract, pathogenic variants are implicated in foodborne illnesses and various infections [17]. Azithromycin (AZM), a macrolide antibiotic structurally related to erythromycin, exerts its antibacterial action primarily by inhibiting bacterial protein synthesis [18,19]. In addition to its antimicrobial effect, AZM exhibits anti-inflammatory and immunomodulatory properties. Nevertheless, its therapeutic potential against Enterobacterales such as *E. coli* is compromised by poor permeability of the membrane and low intrinsic activity [20]. Furthermore, AZM suffers from low oral bioavailability (~36%), high therapeutic dose requirements (typically 500 mg), and dose-related adverse effects such as gastrointestinal distress and cardiovascular symptoms, which hinder patient compliance [21].

This study aimed to explore whether lipid-based nanocarriers can enhance the uptake of azithromycin into bacterial cells, thereby improving its antibacterial performance against E. coli. We also investigated the feasibility of achieving comparable efficacy using lower antibiotic concentrations. Comprehensive physicochemical characterization of the azithromycin-loaded SLNPs was performed, including analysis of particle size, zeta potential, drug release profile, and *in-vitro* antibacterial activity.

Materials and methods

Preparation of solid lipid nanoparticles (SLNPs)

SLNPs were formulated using the hot homogenization technique, following the procedure described by Gazi and Krishnasailaja [22] and further optimized by Helal *et al*. [26]. Briefly, 5 g of glycerol monostearate (solid lipid) was melted at 70 °C, after which 1 g of soya lecithin (lipophilic surfactant) was incorporated and stirred until complete homogeneity. Simultaneously, an aqueous phase was prepared by adding 1.5 mL of Tween 80 to 100 mL of distilled water, which was also heated to 70 °C under continuous stirring for 15 minutes. The molten lipid phase was then added dropwise to the aqueous surfactant phase under high-speed homogenization at 15,000 rpm for 5 minutes. The resulting emulsion was further processed by probe sonication for 15 minutes and stored at room temperature until further use.

Encapsulation of azithromycin into SLNPs

As recommended by Helal *et al*. [26], 0.5 g of azithromycin (AZM) was incorporated into 4.5 g of solid lipid (GMS) and melted at 70 °C along with 1 g of soya lecithin to ensure uniform dispersion. This lipid-drug mixture was then added dropwise to the pre-heated aqueous surfactant solution and homogenized at 15,000 rpm for 5 minutes in a graduate high speed automatic homogenizer for 5 minutes at 15000 rpm and the formulation was then sonicated using automatic high speed sonicator for 15 minutes [22].

Loading of azithromycin on SLNPs emulsion

Surface loading of the drug was performed by adding 20 cm^3 of azithromycin suspension (50 mg ml^{-1}) into 30 cm^3 of SLNPs emulsion. The mixture was magnetically stirred for 6 hours at room temperature to facilitate drug adsorption onto the nanoparticle surface [23, 24].

Characterization of the prepared nanoformulation

Morphological characterization

The morphology and particle size of SLNPs, either singly or AZM-loaded, were examined using transmission electron microscopy (TEM, JEOL 1010, 80 kV, EM Unit, Mansoura University). To prevent aggregation, samples were sonicated for 2 minutes prior to analysis. One drop of each formulation was placed on a carbon-coated copper grid and air-dried. Size measurements were performed using Image-Pro Plus 4.5 software [25–27].

Chemical characterization

Zeta potential

Zeta potential was determined using a Malvern Instruments ltd zetasizer Nano ZS in electron microscope Unit at Mansoura University. The zeta cell was cleaned sequentially with distilled water, ethanol, and distilled water, and dried by gentle nitrogen stream to prevent contamination. One milliliter of each nanoparticle suspension was introduced into the cell *via* syringe, and three independent measurements were taken per sample [26–28].

Fourier transformation infrared spectroscopy (FTIR) analysis

FTIR analysis was conducted to assess potential interactions between AZM and SLNPs. Approximately 3 mg of dried formulation was mixed with 0.1 g of potassium bromide and compressed into pellets under vacuum for 10 minutes. Spectra were recorded using a NICOLET IS10 FTIR spectrometer (Faculty of Science, Mansoura University) as described by Trykowski *et al*. [29].

***In-vitro* drug release studies**

The release profile of AZM from SLNPs was evaluated using the dialysis bag diffusion technique, according to Kashanian *et al*. [30] and Helal *et al*. [26]. AZM-loaded SLNPs were placed in dialysis bags (molecular weight cut-off: 12–14 kDa), which were immersed in 50 cm^3 phosphate buffer (pH 6.8) at 37 ± 1 °C and agitated at 100 rpm. At hourly intervals, 5 cm^3 of the release medium was withdrawn and replaced with fresh preheated buffer. The concentration of released AZM was quantified spectrophotometrically at 270 nm, and cumulative release was calculated using a pre-established standard curve.

Preparation of different AZM-loaded SLNPs concentrations

A stock solution of AZM was prepared in distilled water and serially diluted to achieve final concentrations of 50, 5, 0.05, and 0.005 mg ml^{-1}. These were incorporated into SLNPs emulsions and stirred for 6 hours.

Biological Characterization

Determination of minimum inhibitory concentration (MIC)

The MIC was determined using broth dilution assay. *E. coli* isolates obtained from the Microbiology Laboratory, Faculty of Veterinary Medicine, Mansoura University were cultured, and 1–2 colonies were suspended in 5 cm^3 sterile distilled water. Serial dilutions of AZM-loaded SLNPs (as above) were inoculated with 100 µL bacterial suspension in sterile nutrient broth tubes and incubated at 37 °C for 24 hours. The lowest concentration showing no visible growth was recorded as the MIC [27, 31].

Antibacterial activity evaluation of AZM, SLNPs and AZM-loaded SLNPs

The antibacterial activity of AZM, SLNPs and AZM-loaded SLNPs was assessed using the agar well diffusion method. Sterile nutrient agar (15 cm^3) was poured into sterilized Petri dishes and inoculated with 0.1 cm^3 of bacterial suspension. Wells (1 cm diameter) were punched using a sterile cork borer and filled with 100 µL of each test formulation. After pre-diffusion at room temperature for 45 minutes, plates were incubated at 37 °C for 24 hours. Zones of inhibition were measured in millimeters [27, 32].

Results and discussion

Characterization of the prepared nanoformulations (SLNPs and AZM-Loaded SLNPs)

Physical properties

Morphology and size

Transmission electron microscopy (TEM) was employed to evaluate the morphology and size of the formulated solid lipid nanoparticles, both unloaded and loaded with azithromycin (AZM). As illustrated in Figure 1, both SLNPs and AZM-loaded SLNPs exhibited a semi-spherical morphology. The particle size of the unloaded SLNPs ranged from 59.17 nm to 135.57 nm. Upon loading of AZM, a marked increase in particle size was observed (Figure 1B), with a maximum increase of approximately 61.26% in mean diameter.

These findings are consistent with those reported by Shih and Li [33] and Rosseto *et al*. [34], who successfully synthesized SLNPs displaying near-spherical, amorphous structures with sizes of 57.55 ± 15 nm and 71.97 ± 5 nm, respectively.

Hence, the current investigation focused on

studying the antibacterial potency of SLNPs either alone or in loaded with azithromycin antibiotic. Solid lipid nanoparticles attracted more attention among other polymeric nanoparticle due to their unique physicochemical characteristic [35]. Therefore, the application of AZM-loaded SLNPs should be a worthwhile and promising strategy in drug delivery and diseases treatment.

In the current study, SLNPs were prepared using the hot homogenization method, wherein the lipid (glycerol monostearate) was melted above its melting point and emulsified with soya lecithin (lipophilic surfactant) and Tween 80 (hydrophilic surfactant), in line with previously established protocols [26, 27]. High processing temperatures are known to reduce particle viscosity, potentially resulting in smaller particle sizes; however, they may also accelerate thermal degradation of both the drug and the carrier system. Moreover, excessive homogenization pressures or prolonged processing times can paradoxically increase particle size due to elevated kinetic energy and possible particle aggregation [26, 27, 36].

The observed increase in particle size following AZM loading may be attributed to drug adsorption onto the surface or entrapment within the lipid matrix, resulting in increased nanoparticle volume. This phenomenon has been previously documented by Helal et al. [26], Sabry *et al*. [27], and Alarifi *et al*. [37], who reported similar size increases upon antibiotic incorporation into SLNP systems.

The enhanced drug-loading efficiency observed in the AZM–SLNP system is likely due to the amphiphilic nature of the SLNPs, which provide both hydrophilic and hydrophobic interaction sites for drug molecules. Their large surface area and affinity for hydrophobic drugs facilitate efficient adsorption and incorporation, supporting the morphological observations reported in this study [33]. Furthermore, Hosseini *et al*. [38] reported comparable results with doxycycline-loaded SLNPs, noting spherical morphology, uniform size distribution, and an increase in particle diameter from 258.8 nm to 299.3 nm following drug incorporation.

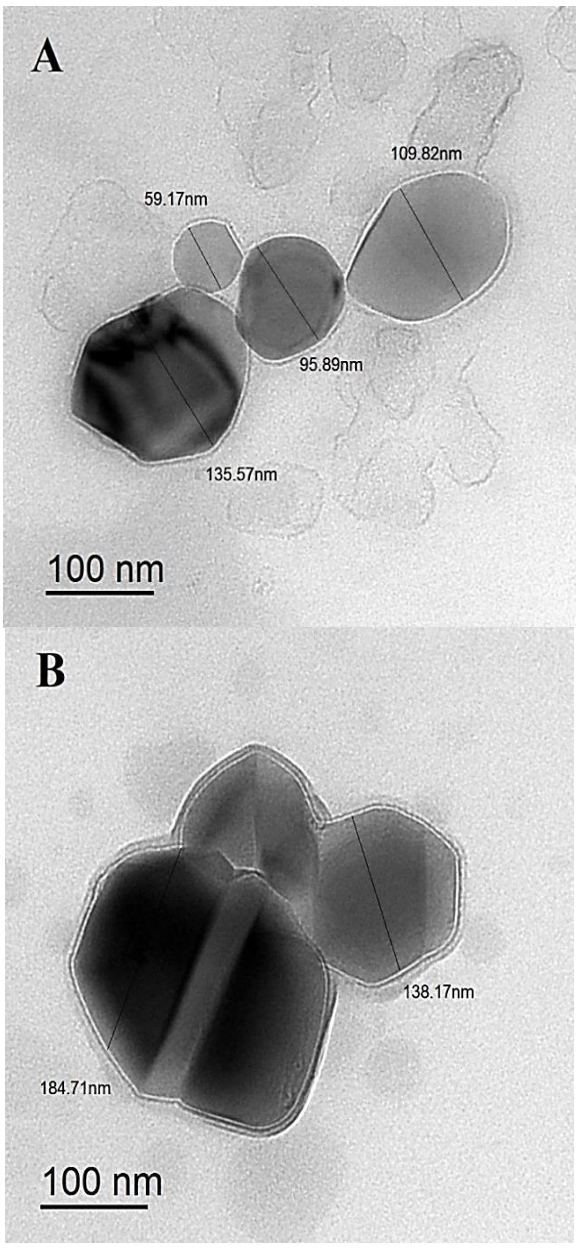

Figure 1: TEM micrograph of **A**; solid lipid nanoparticles (SLNPs) and **B**; azithromycin-loaded solid lipid nanoparticles (SLNPs-AZM).

Chemical properties

Zeta potential

Zeta potential (z p ζ) for SLNPs and SLNPs-AZM were measured to determine the surface charge and confirm the stability of the prepared nanomaterials. As shown in figure 2, SLNPs either singly or in combination with AZM had high negative zeta potential values meaning that SLNPs had a negative surface charge. Furthermore, the value was increased from -21.80 mV to -35.80 mV with the addition of AZM antibiotic indicating the increase of the nanodrug stability.

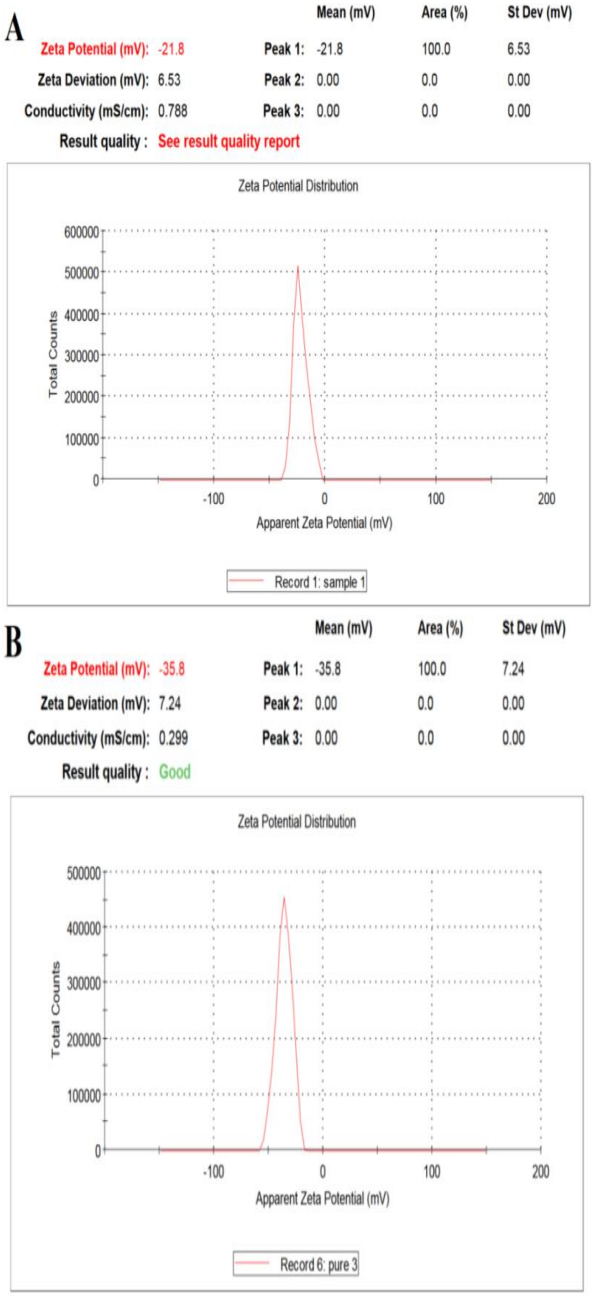

Figure 2: Zeta potential of A; solid lipid nanoparticles and B; azithromycin- loaded solid lipid nanoparticles.

Zeta potential represents the electrical potential difference between the surface of electrophoretically mobile nanoparticles and the surrounding dispersion medium. It serves as a key indicator of colloidal stability, where higher absolute zeta potential values (typically > ±30 mV) suggest strong electrostatic repulsion between particles, thereby reducing aggregation and maintaining suspension stability [39]. In this context, the observed negative surface charge is primarily attributed to lecithin, while Tween 80 may contribute a slight additional negative charge due to its hydrogel properties [40]. Notably, the zeta potential of the nanocarriers increased significantly upon the incorporation of azithromycin (AZM) onto the surface of SLNPs, reflecting improved stability of the formulation.

These results are agreeing with the findings of Helal *et al*. [26] and Sabry *et al*. [27] who reported that zeta potential values of all SLNPs formulations were with negative values and their values were increased when incorporated with nystatin and fluconazole antifungal and ciprofloxacin antibiotic. Also, Said *et al.* [41] reported that rosemary loaded solid lipid nanoparticle had a zeta potential of -33.3 mV, which shows that they are very stable.

FTIR analysis

Fourier-transform infrared (FTIR) spectroscopy is widely employed for molecular characterization, enabling the identification of functional groups to confirm nanoparticle formation. In this study, FTIR was utilized to evaluate the presence of functional groups both SLNPs and AZM-SLNPs, with their spectra presented in Figures 3 and 4.

The FTIR spectrum of the prepared SLNPs (Figure 3) exhibited characteristic absorption peaks including a broad band at 3314 cm^{-1} corresponding to the hydroxyl (–OH) stretching vibration. Peaks observed at 2918 cm^{-1} and 2850 cm^{-1} were attributed to methyl ($–CH_3$) and methylene ($–CH_2$) stretching vibrations, respectively. Additionally, a peak near 1733 cm^{-1} was assigned to the stretching vibration of carboxyl groups.

In Figure 4, the FTIR spectrum of AZM-SLNPs showed no new peaks compared to blank SLNPs, indicating the absence of chemical bond formation between azithromycin and the lipid matrix. The adsorption of AZM onto SLNPs likely involves surface interactions rather than covalent bonding. Functional groups in azithromycin may facilitate secondary interactions such as hydrogen bonding, electrostatic forces, or π-π interactions with the nanoparticle surface.

These findings are consistent with previous studies by Helal *et al*. [26] and Sabry *et al*. [27], who reported similar FTIR spectral patterns for antifungal and antibiotic drugs (nystatin, fluconazole, ciprofloxacin) loaded onto SLNPs.

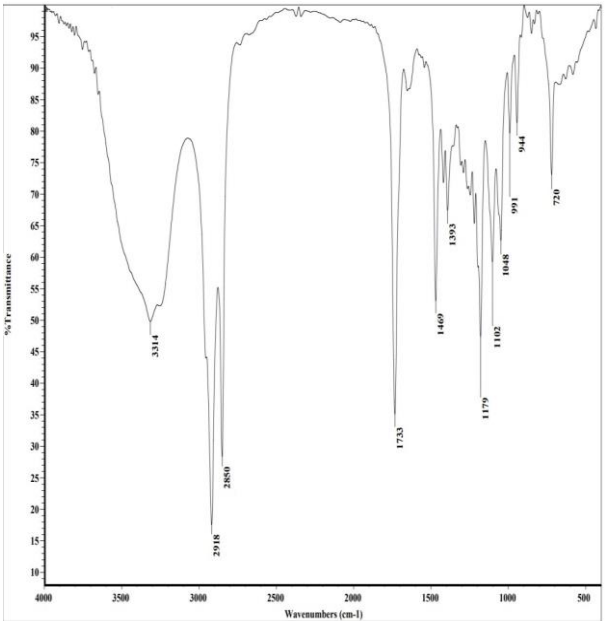

Fig. 3. Infrared (IR) spectrum of solid lipid nanoparticles (SLNPs)

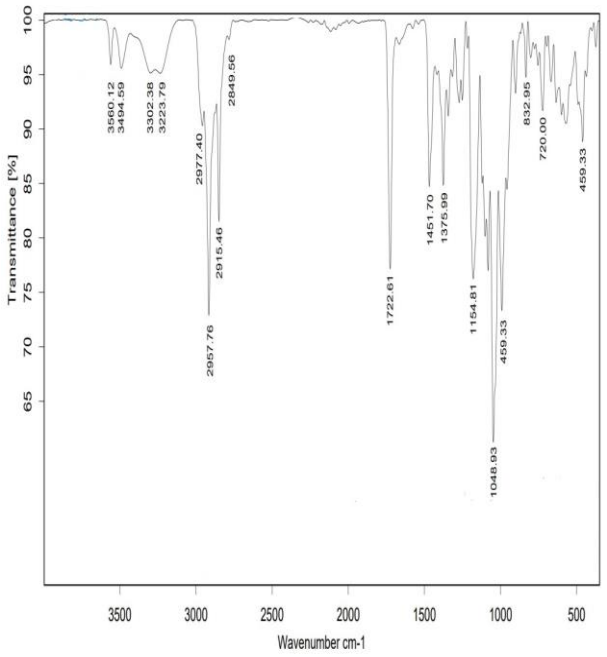

Fig. 4. Infrared (IR) spectrum of azithromycin-loaded solid lipid nanoparticles.

In-vitro drug release study

The *in-vitro* release profile of the drug from the surface of SLNPs exhibited a biphasic release pattern. As illustrated in Figure 7, an initial release was followed by a slower and sustained drug release phase. Within the first 6 hours, approximately 25.8% of the encapsulated drug was released. By the 12-hour mark, the cumulative release reached around 37.2%. Notably, the observed slow and incomplete release pattern may indicate enhanced cellular uptake of the nanoparticles across the bacterial cell membrane.

The observed low drug release was probably due to the hindrance in drug diffusion by the glycerol monostearate matrix, which is composed of compact structure wherein its meshes do not allow flow of liquid phase and hence a significant presence of the drug remained within the SLNPs [42]. Due to their nanoscale size, nanomaterials possess a high surface area that facilitates enhanced drug release [26, 27]. A sustained or slow release profile is typically achieved when the drug is uniformly dispersed within the lipid matrix, which depends on the type of nanomaterial and the drug entrapment model employed [43]. Drug release from polymeric systems can occur through several mechanisms, including (a) surface erosion of the polymer matrix, (b) cleavage of polymer bonds either at the surface or within the bulk matrix, and (c) diffusion of the encapsulated drug [26, 27, 44].

Weng *et al*. [45] described that diffusion-controlled drug release involves the swelling of the system as water penetrates, leading to the transformation of the solid lipid into a rubbery matrix, followed by the diffusion of the drug from this swollen matrix.

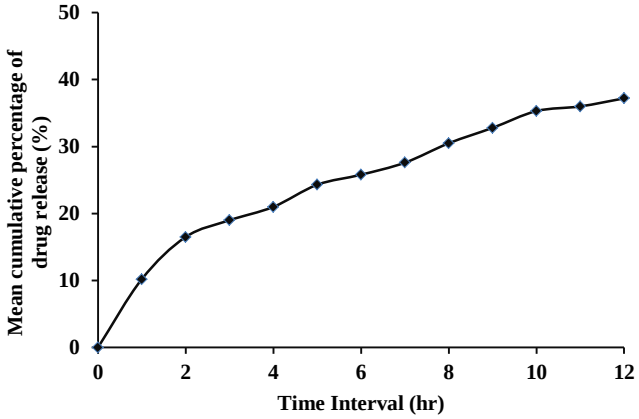

Fig. 5. *In-vitro* drug release profile of azithromycin antibiotic loaded solid lipid nanoparticles.

Biological properties

Determination of minimum inhibitory concentration (MIC)

To ascertain the inhibitory effect of AZM on *E. coli* pathogen, the MIC values for free AZM was determined using microdilution assay. The Various concentrations of AZM exhibited differential inhibitory effects on the growth of *E. coli*. The minimum inhibitory concentration (MIC) was determined to be 0.05 mg mL^{-1}, as illustrated in Figure 6.

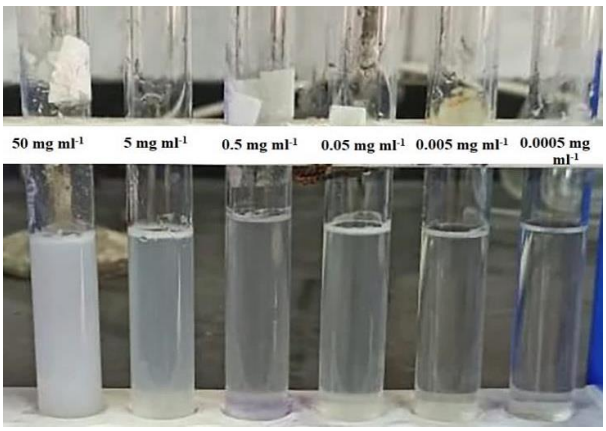

Fig. 6. Representative image illustrating the antibacterial activity of different concentrations of free azithromycin against *Escherichia coli*

Antibacterial efficacy of AZM and SLNPs either singly or in combination

The susceptibility of tested bacterial strain toward free AZM and SLNPs either singly or in combination at different doses was determined following the agar well diffusion method. Table 2 and figure 7 show the antibacterial activity of AZM, SLNPs and SLNPs-AZM in terms of inhibition zone (diameter). There was no significant difference in the zone of inhibition between AZM and SLNPs. However with 50 mg ml^{-1}, the zone of inhibition around wells containing AZT-SLNPs was more significant (9.01 %), than the one containing other doses.

Table 2. Antibacterial efficacy of azithromycin and solid lipid nanoparticles, individually and in combination as a nanocarrier-based delivery system against *Escherichia coli*. Mean values listed are given as mm for the zone of inhibition.

Antibacterial agent	Zone of Inhibition	Percent change (%)
AZM; 50 mg ml^{-1}	**2.2**	--
AZM; 5 mg ml^{-1}	1.5	-31.81
AZM; 0.5 mg ml^{-1}	2.0	-9.09
AZM; 0.05 mg ml^{-1}	0.9	-59.09
SLNPs; 5%	2.0	--
SLNPs; 5%+ AZM; 50 mg ml^{-1} (Encapsulated)	2.0	-59.09
SLNPs; 5%+ AZM; 50 mg ml^{-1} (Loaded)	**2.4**	**9.10**
SLNPs; 5%+ AZM; 5 mg ml^{-1}	1.8	-18.18
SLNPs; 5%+ AZM; 0.5 mg ml^{-1}	2.1	-4.54
SLNPs; 5%+ AZM; 0.05 mg ml^{-1}	1.8	-18.18

Our results are in good agreement with the report on the antibacterial activity of tilmicosin-SLNPs, which could inhibit the microbial growth at smaller concentration than pure drug itself [46]. The low bioavailability of azithromycin indicates that it is poorly absorbed leading to low concentrations in plasma and insufficient amount of drug reaching the site of action. Hence, the beneficial pharmacologic effects of the new chemical entity may not be realized. For this reason, a high dose of AZM (500 mg) is given, which invariably causes adverse effects. The need to lower the concentration and dosing of the drug and to prevent its untoward adverse effects necessitates a carrier-mediated delivery of the drug to infected cells. Colloidal carriers such as liposomes, polymeric nanoparticles, lipid nanocarriers have been investigated and developed as targeted strategies especially for the treatment of intracellular infections [47, 48].

The ability of SLNPs to easily incorporate lipophilic drugs in biocompatible lipid matrix offers advantages in drug delivery, which could be used for drug targeting in the treatment of complex diseases and this can be attributed to the modified surface characteristics as well as increased drug adsorption and uptake [49]. *E. coli* with its thinner peptidoglycan layer and an outer lipopolysaccharide membrane has limited permeability to drugs [50]. Nanostructure with controlled drug release weakens its membrane resistance and increases drug uptake over time.

We hypothesize that the enhanced antibacterial activity of AZM -loaded SLNPs is attributed to their small size and the specific characteristics of their lipid and surfactant components. Previous studies have indicated that lipid-based nanoparticles incorporating Tween 80 exhibit increased antibacterial efficacy against *E. coli* [51]. Additionally, SLNPs may facilitate targeted intracellular delivery of the drug and function as efflux pump inhibitors, thereby reducing the active expulsion of the antibiotic from bacterial cells [52].

In conclusion, our results indicated that solid lipid nanoparticles are good and novel nanodrug delivery system for azithromycin antibiotic and considered the best and suitable

treatment approach for *E. coli* compared with using antibiotic singly as a traditional treatment. The present results need further investigation in particular in the biosafety field.

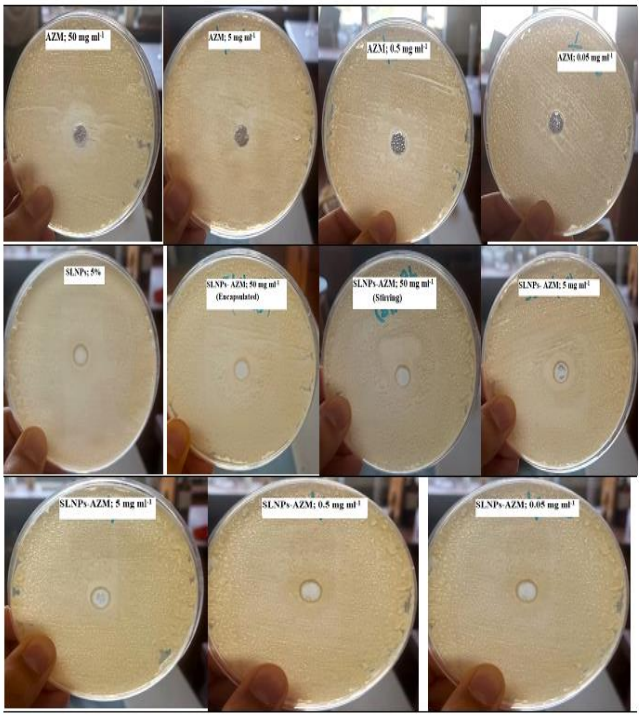

Fig. 7. Antibacterial efficacy of SLNPs and azithromycin-loaded SLNPs against *Escherichia coli.*

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