Formulation and Evaluation of Azithromycin-Loaded Niosomal Gel: Optimization, In Vitro Studies, Rheological Characterization, and Cytotoxicity Study

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ABSTRACT: Several novel, innovative approaches for improving transdermal delivery of BCS class III drugs have been proposed. Despite their great aqueous solubility, BCS class III drugs have the drawback of limited permeability. The objective of the current work was to screen the suitability of niosomes as a nanocarrier in permeation enhancement of azithromycin (AZM) transdermal delivery. Niosomes were prepared by an ether injection method using a nonionic surfactant (Span 60) and cholesterol at different concentrations. The ζ potential (ZP), polydispersity index (PDI), and particle size (PS) of AZM-loaded niosomes were evaluated. The size of the niosomes was found to vary between 288 and 394 nm. The results revealed that the niosomes prepared in a ratio of 2:1 (Span 60: cholesterol) had larger vesicle sizes, but all of them were characterized by narrow size distributions (PDI <0.95). Niosomal gel was successfully prepared using different polymers. The appearance, pH, viscosity, and ex vivo drug release of niosomal gel formulations were all examined. The flow curves showed that the niosomal gel displayed lower viscosity values than its corresponding conventional gels. Niosomal and conventional gels exhibited a domination of the elastic modulus (G′) over the viscous modulus (G″) (G′>G″) in the investigated frequency range (0.1−100 rad/s), indicating stable gels with more solid-like properties. Ex vivo skin permeation studies for the niosomal gel show 90.83 ± 3.19% of drug release in 24 h as compared with the conventional gel showing significantly lower (P < 0.001) drug release in the same duration (1.25 ± 0.12%). Overall, these results indicate that niosomal gel could be an effective transdermal nanocarrier for enhancing the permeability of AZM, a BCS class III drug. In conclusion, this study suggests that transdermal formulations of AZM in the niosomal gel were successfully developed and could be used as an alternative route of administration.

1. INTRODUCTION

Transdermal drug delivery (TDD) is one of the most convenient systems for patients. TDD has many advantages such as being painless, easy to apply, avoiding the first-pass effect, and having a better pharmacokinetic profile for extended periods and fewer side effects.1 Permeation of drugs across the stratum corneum (SC) is the rate-limiting step for transdermal delivery.2 Several nanocarriers have emerged and are being used to improve the pharmacokinetic and pharmacodynamic profiles of drugs. Therefore, vesicular systems have been used to increase the absorption of encapsulated drugs while also providing controlled and sustained therapeutic activity.2,3 Several forms of vesicular systems have been studied for drug administration, including liposomes, phytosomes, bilosomes, niosomes, and exosomes.4−6

Niosomes have an amphiphilic bilayer structure with a polar part on the surface and a non-polar zone inside and are made up of nonionic surfactants and cholesterol.7 Niosomes can be unilamellar or multilamellar and can carry both hydrophilic and lipophilic drugs while also delivering them to the target site.7−9 Because of their greater chemical stability and reduced cost, niosomes are regarded as one of the promising alternatives to liposomes.7,8 Pharmaceutical nanocarriers such as niosomes have been popular in the field of transdermal drug delivery due to their unique advantages like enhancing skin penetration, providing a controlled and sustained release, showing better drug stability, ability to encapsulate both hydrophilic and hydrophobic drugs, and lower cost compared...
with other nanocarriers.\textsuperscript{7,8,10,11} Niosomes also have a number of advantages, including minimal toxicity, and are relatively simple to manufacture.\textsuperscript{10,12} As a result, niosomes have been used to improve the drug permeability of AZM.

Azithromycin (AZM) is a macrolide antibiotic that can be taken orally, parenterally, or as an ophthalmic solution.\textsuperscript{13} AZM is commonly used to treat infections of the upper and lower respiratory tract infections.\textsuperscript{13} It is well known for its effectiveness in treating skin infections in both adults and children.\textsuperscript{13−15} Azithromycin’s clinical usefulness in dermatology is not confined to infectious illnesses.\textsuperscript{13,16} In addition to its antibacterial properties, AZM appears to be safe to use in patients with dermatological conditions such as persistent rosacea, psoriasis, and acne because of its immunomodulatory and anti-inflammatory properties.\textsuperscript{15−17} In addition, it has a poor bioavailability of 37%, a relatively long half-life of up to 96 h, and is broadly dispersed in tissues throughout the body.\textsuperscript{18−20} The most typically reported side effects of oral administration of AZM are abdominal discomforts such as diarrhea, abdominal pain, nausea, and vomiting.\textsuperscript{19,20} An alternative administration route would make a significant contribution to the patients. Therefore, developing new AZM delivery systems with a higher bioavailability and a lower dose will have a significant therapeutic impact on reducing gastrointestinal (GI) adverse drug effects and overall improve patients’ compliance. AZM is classified as a biopharmaceutical classification system (BCS) class III, which has low permeability.\textsuperscript{21} The encapsulation of AZM in niosomes is expected to improve drug permeability and allow for longer drug release. AZM-loaded niosomes might provide new opportunities to improve the transdermal delivery of BCS class III drugs.

A niosome-based gel system is a nanotechnology-mediated drug delivery technique that is thought to be an effective tool for improving the biopharmaceutical properties of class III drugs. The aim of this study was to evaluate the ability of niosomes to facilitate the transdermal delivery of AZM and to identify the best niosomal formulation based on the assessment of their size and entrapment efficiency. After that, niosomes were loaded into the gel system and evaluated.

2. MATERIALS AND METHODS

2.1. Materials. Azithromycin was purchased from Sigma-Aldrich (St. Louis, MO, USA). HPLC-grade acetonitrile, methanol, diethylether, isopropanol, and HPLC-grade water were purchased from Tedia (Fairfield, USA). Span 60, cholesterol, sodium carboxy methyl cellulose (Na-CMC), sodium hydroxide, and phosphate buffered saline (PBS) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Carbopol 974 was purchased from Lubrizol (Ohio, USA). The 4,5-(dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium substrate and dimethylsulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Methods. 2.3. Preparation of Niosomes. Different niosomal formulations were prepared by an ether injection method using a nonionic surfactant (Span 60) and cholesterol at different concentrations as shown in Table 1. Briefly, Span 60 and cholesterol were dissolved in (16 mL) diethylether, and AZM was dissolved in (4 mL) methanol. Then, the resulting solution was slowly injected using a microsyringe at a rate of 1 mL/min into 20 mL of hydrating solution PBS (pH 7.4). The solution was stirred continuously on a magnetic stirrer, and the temperature was maintained at 60−65 °C. As the lipid solution was injected slowly into the aqueous phase, the differences in temperature between phases caused rapid vaporization of ether, resulting in spontaneous vesiculation and formation of niosomes. The proportion of the drug, surfactant, and cholesterol for the preparation of niosomes is given in Table 1. The ratios of cholesterol and surfactants used in the development of niosomes were chosen based on previous studies.\textsuperscript{13} F2 and F3 are in the same ratio, but different amounts of cholesterol and Span 60 were used. All niosomal dispersions were stored in a refrigerator at 4 °C. Blank niosomes were prepared under the same conditions but without the addition of AZM and were used as controls to study the effect of the loading drug on the particle size and for ATR-FTIR analysis.

2.4. Evaluation of Niosomes. 2.4.1. Transmission Electron Microscopy (TEM). The morphology of niosomes was observed using a transmission electron microscope (TEM, FEI Morgani 268, operating voltage of 60 kV, Holland) connected to a Megaview II digital camera. One drop of niosomes was diluted with distilled water (1:2 v/v), placed on a carbon-coated copper grid, and allowed to dry before imaging. The morphology of niosomes was estimated using ImageJ software.

2.4.2. ζ Potential and Particle Size Measurements. The ζ potential (ZP), polydispersity index (PDI), and particle size (PS) of AZM-loaded niosomes were determined using a particle Zetasizer analyzer (Brookhaven 90 plus, USA). The surface charge of the particles was measured by the electrophoretic light scattering (ELS) technique. The polydispersity index (PDI) was automatically displayed using the internal Zetasizer software for all of the range of particles analyzed. Each experiment was performed in triplicate, and the results were presented as mean ± SD.

2.4.3. Determination of Entrapment Efficiency. The unentrapped drug was separated from the niosomes by ultracentrifugation. Briefly, 1.5 mL of each niosome suspension was ultracentrifuged (Beckman Optima LE-80 K ultracentrifuge, Beckman Coulter Inc, Fullerton, CA) for 1 h at 16,000 rpm at 4 °C. The supernatant (unentrapped drug) was removed, and the niosomes were washed three times with PBS and ultracentrifuged under the same conditions. The content of the entrapped drug was determined after dissolving 0.1 mL of the niosomes in 1 mL of isopropanol until they were clear and then diluted up to 10 mL with PBS; after that, the samples were sonicated for 5 min at room temperature (RT). The amount of entrapped AZM was determined by HPLC. The EE % was calculated using the following eq 1:

\[
EE(\%) = \left( \frac{\text{Amount of drug entrapped}}{\text{Total AZT amount}} \right) \times 100\%
\]

where the amount of drug entrapped refers to the actual amount of drug molecules that have been successfully encapsulated in the vesicles, and the total AZM amount refers to the amount of AZM that was used at the beginning of the preparation.

### Table 1. Composition of AZM-Loaded Niosomes

| Formula | AZM (mg) | Span 60 (mg) | Cholesterol (mg) |
|---------|----------|--------------|------------------|
| F1      | 200      | 200          | 200              |
| F2      | 200      | 200          | 100              |
| F3      | 200      | 400          | 200              |

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Table 2. Composition of Gel Formulations With Different Polymers

| ingredients       | NG-1 (w/w) | NG-2 (w/w) | CG-1 (w/w) | CG-2 (w/w) |
|-------------------|------------|------------|------------|------------|
| Carbopol 974      | 0.5%       | 0.25%      | 0.5%       | 0.25%      |
| Na-CMC            | 0          | 3%         | 0          | 3%         |
| azithromycin      | 0.5%       | 0.5%       | 0.5%       | 0.5%       |
| water             | q<sub>s</sub> | q<sub>s</sub> | q<sub>s</sub> | q<sub>s</sub> |

2.5. Short-Term Stability Studies. A short-term stability study was investigated in terms of PS, PDI, and EE %. AZM-loaded niosomes (F1, F2, and F3) were stored at 4 °C and then, their PS, PDI, and EE % were determined after 3 months.

2.6. In Vitro Release Studies. The in vitro release of AZM from niosomes suspension (previously separated from the unentrapped drug by ultracentrifugation) was carried out using Franz diffusion cells under sink conditions and a heating circulator set to 37 °C. A dialysis membrane was washed and soaked in PBS. The receiver compartment (12 mL) was filled with PBS and 10% (v/v) isopropanol, which was added to ensure sink conditions. Then, a sample of niosomes (corresponding to 3 mg of AZM) was added to the donor chamber on a pre-soaked cellulose membrane, and the system was adequately sealed. The receptor medium was constantly mixed with a magnetic stirrer. Aliquots (1 mL) were withdrawn from the receiver compartment at certain time intervals (1, 2, 3, 4, 5, and 24 h) and replaced with the fresh medium. The amount of released AZM was determined by HPLC. The cumulative amount of AZM permeated through the membrane (Q) was plotted versus time (t). The steady-state flux (J<sub>ss</sub>, μg/cm²/h) was calculated from the slope of the linear portion of the cumulative amount of AZM permeated per unit area (Q/A) versus time plot. Apparent permeability (P) was calculated according to eq 2:

\[ P = \frac{J_{ss}}{C_0} \]  

where Co is the drug concentration in the donor solution. Under sink conditions, the concentration of the drug in the receptor compartment is assumed to be negligible compared to the concentration of the drug in the donor compartment.

2.7. Cytotoxicity. The cytotoxicity of the niosomal formulations F2, F3, and free drug (AZM) was determined using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2-H-tetrazolium bromide (MTT) assay. F1 was excluded due to its low EE %. The Michigan Cancer Foundation-7 (MCF-7) cell line was maintained in (RPMI) medium containing 10% fetal bovine serum at 37 °C in a humidified atmosphere with 5% carbon dioxide (CO₂). MCF-7 cells were seeded in a 96-well plate at a density of 5000 cells/well. The cells were incubated for 24 h to allow for cell attachment and recovery. On the following day, 20 μL of different serial dilutions (100, 10, 1, and 0.1 μM) of each formulation and AZM were added to the appropriate wells and incubated for 72 h at 37 °C. Cisplatin was used as a positive control at 10 μM. After treatment, 20 μL of a 5 mg/mL solution of the 4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) tetrazolium substrate was added to each well and incubated for 4 h at 37 °C. The medium was removed, and the resulting violet formazan crystals were then solubilized by the addition of DMSO. The plates were then analyzed on a microplate reader (GloMax) at 450 and 570 nm to determine the absorbance of the samples. Cell survival (%) versus drug concentration (μM) was plotted into excel spreadsheets. Each condition was repeated in quadruplicate in three independent experiments for each cell line.

2.8. Antimicrobial Efficacy Evaluation. The antibacterial activity of AZM niosomal formulation was measured using inhibitory zone measurements against Gram-negative and Gram-positive organisms, including *Staphylococcus aureus* (ATCC 6538) and *Staphylococcus epidermis* (ATCC 35218). The agar well plate diffusion method was used to ensure the antimicrobial properties of niosomal formulations, according to the Clinical and Laboratory Standards Institute (CLSI) for bacteria and yeast testing. The wells (9 mm in diameter) were prepared by using a sterile cork borer, and an overnight bacterial inoculum was uniformly spread using a sterile cotton swab over a sterile Mueller–Hinton agar plate. The niosomal formulation (F2) (equivalent to 15 μg) and the AZM working standard solution (15 μg, potency 94.4%, assay 98.9%) were transferred into the wells and incubated for 24 h at 37 °C. After incubation, confluent bacterial growth was observed. Inhibition of the bacterial growth was measured in mm. The positive and negative controls for the observation of growth promotion were also performed.

2.9. ATR-FTIR Analysis. Attenuated total reflectance—Fourier transform infrared spectroscopy (ATR-FTIR) was conducted using a Perkin Elmer UATR-II. Spectra were acquired in the absorbance mode; resolution was set to 2 cm<sup>−1</sup> with 32 scans per sample. The acquired spectral data were exported in the .CSV format and analyzed using Spectragryph Version 1.2.15.

2.10. Fabrication of Niosomal Gels. For the fabrication of gels, the niosomal formulation with the highest in vitro release rate and EE % (F2) was used. The niosomal gel of the chosen formula (F2) was formulated by incorporating the niosomal dispersion in a mixture of Carbopol 974 gel base and Na-CMC. Briefly, a sufficient amount of Carbopol 974 was gradually added to water and kept for 24 h for complete hydration of polymer chains. Na-CMC was added slowly to the above gel base under continuous stirring. The chosen AZM-loaded niosomes (F2) were added slowly to the hydrated Carbopol 974 and Na-CMC mixture to obtain the niosomal gel with a final concentration of 0.5% (w/w) of AZM for all formulations. Then, triethanolamine (TEA) was utilized to cause gelling to adjust the pH of the prepared gel to pH 6.5 using a pH meter. Besides, in a similar manner, conventional gels were also formulated with the addition of AZM as the free drug: conventional gel-1 (CG-1) and conventional gel-2 (CG-2), as summarized in Table 2. The gels were evaluated for organoleptic properties, homogeneity, pH measurement, and rheological properties.

2.11. Rheological Studies. The rheological studies (viscosity and viscoelastic measurements) of AZM-loaded niosomal gels (NG-1 and NG-2) and AZM conventional gels (CG-1 and CG-2) were performed in triplicate at 32 °C using a controlled-stress rheometer (CSR) (Anton Paar, MCR 302; Graz, Austria) with a cone-plate geometry (gap of 0.1 mm, cone diameter of 25 mm, and cone angle of 1°) as described.
2.11.1. Viscosity Measurement. Gel samples of 0.5 g were loaded onto the plate and left to relax for ~1 min prior to testing. The viscosity of each gel was determined as a function of the applied shear rate ranging from 0.1 to 100 s\(^{-1}\). The flow curves of gels were mathematically evaluated using the Carreau–Yasuda model, using eq 3 to determine the shear-thinning fluids, and the Casson model, using eq 4 to determine the yield stress as described:\(^{28}\)

\[
\frac{\eta - \eta_\infty}{\eta^0 - \eta_\infty} = \left[ 1 + (\lambda\gamma)^{n-1} \right]^{1/a} \tag{3}
\]

where \(\eta\) represents the apparent viscosity at a given shear rate (Pa.s), \(\eta^0\) is the zero-shear rate viscosity (Pa.s), \(\eta_\infty\) is the infinite viscosity (Pa.s), \(\gamma\) is the shear rate (s\(^{-1}\)), and \(\lambda, \alpha, \) and \(n\) are the shape parameters.

\[
\sqrt{\tau} - \tau_0^{1/2} = \eta_\infty^{1/2} \gamma^{1/2} \tag{4}
\]

where \(\tau\) and \(\tau_0\) are the shear stress (Pa) and the yield stress (Pa), respectively. \(\eta_\infty\) is the infinite shear viscosity (Pa.s), and \(\gamma\) is the shear rate (s\(^{-1}\)).

2.11.2. Viscoelastic Measurement. Initially, the strain-sweep measurements for all gels were performed to determine the linear viscoelastic (LVE) region. In this region, the elastic \((G'\)\) and viscous \((G''\)\) moduli of gels must remain constant and independent of strain.\(^{29}\) The LVE region is important since the frequency-sweep measurements of gels must be performed within this region. To determine the LVE region, gel samples of 0.5 g were loaded onto the lower plate and left to equilibrate at 32 °C for 1 min. The upper cone was lowered until the gap between the cone and plate was 0.1 mm; then, the cone was oscillated at a frequency of 6.28 rad/s during measurement. The \(G'\) and \(G''\) moduli were probed as a function of oscillatory strain ranging between 0.01 and 100% as described.\(^{30}\) Based on the strain-sweep measurement, the frequency-sweep measurement was conducted to determine the viscoelastic behavior \((G'\) and \(G''\)\) of the gels. Gels were exposed to dynamic oscillatory strain over a frequency range of 0.1–100 rad/s and at a fixed strain selected from the previously determined LVE region.\(^{28}\)

2.12. Ex Vivo Study. The permeation of AZM from niosomal gel was evaluated using a diffusion cell apparatus (PremeGear, USA) with an orifice diameter of 15 mm and a diffusion surface area of 1.76 cm\(^2\). Conventional gels were used as references to study the effect of niosomal formulations on drug diffusion. The volume of the receiving phase was 12 mL. Full-thickness skin was taken from the back of the rat. The subcutaneous tissue was removed, and the excised skin was rinsed with water, cut into appropriately sized pieces, and frozen at −20 °C. Prior to the experiments, the skin was defrosted and pre-equilibrated in PBS (pH 7.4) for 15 min. The skin was sandwiched between the donor and receptor compartments of Franz cells (surface area of 1.76 cm\(^2\)), with the stratum corneum (SC) facing the donor compartment. The receptor compartment was filled with 12 mL of PBS (pH 7.4) and 10% of isopropanol to maintain sink conditions. It was then continuously stirred on a small magnetic stirrer (600 rpm) with the temperature controlled at 37 ± 1 °C throughout the experiments. Samples of the AZM-loaded niosomal gels NG-1 and NG-2 (corresponding to 10 mg of azithromycin) and control (CG-1 and CG-2) were placed in the donor compartment and properly sealed. A volume of 1 mL of the receptor medium was removed at certain time intervals (1, 2, 3, 5, 7, and 24 h) and immediately replaced with an equal amount of fresh buffer. The drug content in all collected samples was determined by HPLC. The experiments were performed in triplicate.

2.13. HPLC Assay for AZM. Separation was carried out on a Kinetex 2.6 mm XB-C18 column (2.1 mm × 100 mm, Phenomenex, Torrance, USA) using a mixture of acetonitrile and monobasic potassium phosphate buffer prepared by dissolving 4.6 g of anhydrous monobasic potassium phosphate in 900 mL of water. The mixture was adjusted with 1N sodium hydroxide to pH 7.5 and diluted with water to 1L (65/35, v/v) as the mobile phase. All samples were filtered through 0.22 µm Minisart RC4 filters (Sartorius AG) prior to analysis. The flow rate was 0.4 mL/min, and the oven temperature was maintained at 40 °C. UV detection was performed at a wavelength of 210 nm. The chromatographic system consisted of Prominence-i LC-2030C 3D plus and LabSolution software version 5.98 (Shimadzu, Kyoto, Japan). The AZM peaks were detected at a retention time of 3.18 min. Solutions of AZM in methanol at drug concentrations ranging from 0.1–1 µg/mL were used to construct a standard curve \((R^2 = 0.9971)\). Quantitative analysis was performed in triplicate.

2.14. Statistical Analysis. Statistical analyses of the results were performed with a t-test analysis for the cytotoxicity study and in vitro release or one-way analysis of variance (ANOVA) for PS, PDI, ZP, EE %, and the ex vivo study. In each case, \(p < 0.05\) was taken to represent a statistically significant difference.

3. RESULTS AND DISCUSSION

3.1. Evaluation of Niosomal Formulations. In this study, niosomes have been formulated and evaluated as transdermal delivery carriers for AZM. Various formulations of AZM-loaded niosomes were successfully prepared using the ether injection method, as shown in Table 1. Niosomal formulations were then characterized for shape, size, PDI, ZP, and EE %. The TEM micrographs of niosomes are given in Figure 1. TEM was employed to characterize niosomes in terms of shape. The TEM image illustrates that niosomes were of a uniform spherical shape dispersed in the aqueous phase Figure 1. The TEM images confirmed the formation of niosomes.

The mean size of niosomes is influenced by the membrane composition.\(^{31,32}\) Span 60 is a nonionic surfactant with a large hydrophobic moiety characterized by low water solubility (HLB = 4.7).\(^{31,32}\) The PS, PDI, and ZP of NPs are shown in Table 3. The size of the niosomes was found to vary between 288 and 394 nm with no significant difference \((P \text{ value} = 0.0627)\) between them. The results revealed that the niosomes prepared at a ratio of 2:1 (Span 60: cholesterol) \((F2)\) had larger vesicle sizes. The size of drug-loaded niosomes was
found to be significantly higher ($p < 0.05$) than that of unloaded niosomes for each ratio, for example, PS for AZM-loaded niosomes (F2) and the blank F2 formulation was $394.33 \pm 72.25$ and $326.66 \pm 34.00$, respectively. Figure 2 illustrates the intensity-particle size distribution profile for F2 niosomal formulation. F2 and F3 are in the same ratio, but different amounts of cholesterol and surfactants were used. However, there was no significant difference in PS, PDI, and ZP, but EE % was significantly decreased by increasing the amount of cholesterol and Span 60. All niosomal formulations were characterized by narrow size distributions (PDI <0.95).

Shilakari et al.\textsuperscript{33} reported a similar pattern in particle size for drug-loaded niosomal formulations. The temperature of the hydration medium should be greater than the transition temperature of the surfactant, as this influences the formation of niosomes and may cause bilayer instabilities.\textsuperscript{8,34} Therefore, we need to work at temperatures above the transition temperature of the surfactant when forming niosomes to ensure that the ingredients have enough mobility to align appropriately and produce a bilayer lipid vesicle of the correct size/structure. The addition of cholesterol to lipid bilayers improves their stability and shifts the phase transition from the gel to liquid ordered.\textsuperscript{11,35} Cholesterol alters the fluidity of chains in bilayers, thus increasing the breadth of the lipid bilayer, and as a result, the vesicle size increases.\textsuperscript{9,35} Similarly, this has been found to be the case of tenofovir disoproxil fumarate niosomes, that is, when the cholesterol concentration increases, vesicle size also increases.\textsuperscript{36} In addition, they reported that vesicles with surfactant–cholesterol ratios of 1:1 with each grade of Span 60 had higher entrapment efficiency and the highest drug release (99%) after 24 h.\textsuperscript{36} The $\zeta$ potential (ZP) values were $8.75 \pm 0.00$ mV for F1, $14.65 \pm 0.00$ mV for F2, and $11.25 \pm 0.00$ mV for F3. On the other hand, the blank niosomes of F2 had a ZP of $34.50 \pm 0.00$ mV.

The centrifugation method was used to quantify the EE of the niosomal formulations, and the results are shown in Table 3. EE % is an essential characterization parameter that indicates the vesicle's drug encapsulation capacity. It is computed by employing the HPLC analytical method to determine the quantity of the free drug. The EE % was $45.00 \pm 7.45$, $94.60 \pm 16.90$, and $61.33 \pm 1.15$% for F1, F2, and F3, respectively. F2 showed the highest drug encapsulation efficiency (94.60%), and it was significantly higher ($p < 0.05$) than that of other formulations. EE is affected by the vesicle size, surfactant type, alkyl chain length, and cholesterol quantity.\textsuperscript{7,10,37} A niosome vesicle would be composed of nonionic surfactants with varying HLB values that influence EE, which is also stabilized by adding the appropriate quantity of cholesterol to form stable vesicles.\textsuperscript{10,38} In our study, the EE of drug-loaded niosomal formulations improved as the cholesterol–surfactant

| formulation | PS (nm) | PDI     | ZP (mV) | EE %    |
|-------------|---------|---------|---------|---------|
| F1          | 343.00 ± 15.94 | 0.35 ± 0.00 | 29.50 ± 0.00 | 45.00 ± 4.54 |
| F2          | 394.00 ± 72.25 | 0.01 ± 0.00 | 16.80 ± 0.00 | 94.60 ± 16.90 |
| F3          | 288.00 ± 12.00 | 0.34 ± 0.00 | 20.96 ± 0.00 | 61.33 ± 1.15 |

Figure 2. Intensity–particle size distribution profiles for the formulated niosomes (F2).
ratio increased up to a certain limit. Because the usual bilayer structure is disrupted, increasing the cholesterol level beyond a certain point lowers the EE. This could be due to a variety of factors, including an increase in the cholesterol ratio, an increase in the hydrophobicity and stability of the bilayer vesicles, and a decrease in permeability, which could allow the drug to be trapped efficiently in the bilayers as the vesicles develop. Balakrishnan et al. showed that greater levels of cholesterol may compete with the drug for the packing space within the bilayer, preventing the drug from being entrapped. In another study conducted by Farmoudesh et al., naltrexone hydrochloride-loaded niosomes were prepared for ocular delivery. This study showed that the EE % of niosomes containing different nonionic surfactants was reduced when the surfactant–cholesterol ratio was increased from 1:1 to 1:1.4. From the data, niosomal formulations having the highest EE % (F2 and F3) were selected for further studies.

3.2. Stability Study. The stability study of AZM-loaded niosomes after 3 months for PS, PDI, and EE % is displayed in Table 4. The original size of F2 was nearly unchanged after 3 months of storage in its original dispersion at 4 °C. The PS for F1 and F3 was increased significantly (p < 0.05) from 340.33 ± 15.94 nm to 443.88 ± 47.02 nm for F1 and from 288.00 ± 12.00 nm to 348.33 ± 31.53 nm for F3, whereas the PS for F2 was not significantly changed (p > 0.05). These findings suggest that F2 niosomes were stable physically since there were no significant changes in terms of particle size and PDI. Furthermore, evaluating the EE % of the formulation by repeating the experiment after 3 months showed that there were no significant changes (P < 0.05) in the EE % value, indicating that niosome vesicles were maintained during the storage period. For F1, the EE % was 45.00 ± 4.54 and 45.00 ± 4.50% for 0 and 3 months, respectively. For F2, the EE % was 94.60 ± 16.90 and 89.47 ± 2.50% for 0 and 3 months, respectively. For F3, the EE % was 61.33 ± 1.15 and 57.33 ± 9.10% for 0 and 3 months, respectively. Nasser found that niosomes produced from Span 60 with 50 mol % cholesterol inclusion are the most stable over a wide range of temperatures, which was in agreement with our findings. Results showed that the niosomal formulation (F2) was stable at refrigeration in terms of PS, PZ, and EE %. Similarly, for niosomes, a 1:1 ratio of cholesterol to nonionic surfactants in the bilayers is shown to be an optimum ratio for the formation of physically stable niosomal vesicles.

3.3. In Vitro Release. Figure 3 shows the release profiles of AZM from the selected formulations of niosomes (F2 and F3) over 24 h. Interestingly, all the niosomal formulations exhibited sharply increased release of AZM up to 24 h. The release percentage of AZM from F2 was significantly higher than that from F3 after 24 h (p < 0.05). After 24 h of testing, 3.22 ± 0.38 mg of the entrapped drug was released from F2, whereas 2.50 ± 0.64 mg was released from F3. This might be due to the higher EE % of F2 compared with F3. In particular, the cumulative amount (Q/A) of AZM released across the membrane per unit surface area for F2 and F3 after 4 h was

| Table 4. Storage Stabilities of AZM-Loaded Niosomes (PS, PDI, and EE %). The Values are Presented as Mean ± SD (n = 3) |
|---------------------------------------------------------------|
|                  | PS (nm)                  | PDI          | EE %               |
|                  | 0 month | 3 months | 0 month | 3 months | 0 month | 3 months |
| F1                | 340.33 ± 15.94 | 443.88 ± 47.02 | 0.35 ± 0.00 | 0.33 ± 0.00 | 45.00 ± 4.54 | 45.50 ± 4.50 |
| F2                | 394.33 ± 72.25 | 345.80 ± 70.25 | 0.01 ± 0.00 | 0.00 ± 0.00 | 94.60 ± 16.90 | 89.47 ± 2.50 |
| F3                | 288.00 ± 12.00 | 348.33 ± 31.53 | 0.34 ± 0.00 | 0.34 ± 0.01 | 61.33 ± 1.15 | 57.33 ± 9.10 |

Figure 3. In vitro release of AZM from AZM-loaded niosomes F2 and F3 (n = 3).

1089.00 ± 79.20 and 924.14 ± 20.17 μg/cm², which accounts for 39 and 15% of the actual loading of AZM in niosomes, respectively. The permeability coefficient (P) of AZM for F2 and F3 was 0.63 and 0.53 cm/h, respectively, while the flux (Jss) of F2 and F3 was 159.39 and 134.87 μg/cm²/h, respectively.

3.4. Cytotoxicity Assay. In order to evaluate the cytotoxicity of niosomal ingredients of F2 and F3, the MTT assay was performed. It has been shown in recent studies that AZM produces an antiproliferative effect on colon cancer. Therefore, we decided to evaluate if AZM or any of the new niosomal formulations produced an antiproliferative effect on the MCF-7 breast cancer cell line using the MTT assay. Our results showed that neither AZM nor the new formulations have any antiproliferative effects at the tested concentrations as shown in Figure 4. Furthermore, F2 and F3 showed no increased toxicity against AZM, implicating the safety of these formulations.

Figure 4. Cytotoxicity assay of F2, F3, and control AZM on MCF-7.

3.5. Antimicrobial Assay. The antimicrobial effectiveness of the niosomal formulation (F2) containing AZM was evaluated by determining the zones of inhibition of S. aureus ATCC6538 and Staphylococcus epidermidis after 24 h in bacteria culture. The results and zones of inhibition were presented in Table 5 and Figure 5. The encapsulation of AZM in niosomes showed comparable antibacterial activities to free AZM niosomes against S. aureus and S. epidermidis. Furthermore, drug-free niosomes exhibited no antibacterial
activity. Based on these results, the niosomal formulation did not retard the antimicrobial activity of AZM. The zone of inhibition of AZM solution, negative control (NC), positive control (PC), and AZM-loaded niosomes (test item) are shown in Figure 5, which was taken by the authors.

### 3.6. ATR-FTIR

Figure 6 below shows the spectra of the raw materials (Span 60, cholesterol, and AZM), along with the physical mix, blank niosomes, and drug-loaded niosomes.

Characteristic peaks of the spectrum of Span 60 were the peaks seen at 3389, 2916, and 1736 cm\(^{-1}\), likely corresponding to the OH stretching peak, carbonyl dimer, and C═O stretching peak, respectively. A peak characteristic of cholesterol was seen at 3435 cm\(^{-1}\), likely corresponding to OH stretching. Peaks characteristic of AZM were seen at 3559, 3491, 2917, and 1720 cm\(^{-1}\); the two peaks seen above 3500 cm\(^{-1}\) likely correspond to OH stretching, with the other two peaks corresponding to the carbonyl dimer and C═O, respectively.

In the spectrum of the physical mix, the AZM OH stretching was clearly visible at 3560 cm\(^{-1}\), and carbonyl stretching was clearly visible at 1721 cm\(^{-1}\). Furthermore, a broad peak centered around 3477 cm\(^{-1}\), exhibiting a shoulder at approximately 3400 cm\(^{-1}\), was seen in the spectrum of the physical mix, which is most likely a product of the co-elution of the OH stretching peaks of AZM, Span 60, and cholesterol. Furthermore, the C═O stretching peak was visible at 1737 cm\(^{-1}\).

In the spectrum of the blank niosomes, the OH stretching peak of Span 60 was visible at 3388 cm\(^{-1}\). The carbonyl dimer was seen to shift to 2918 cm\(^{-1}\), and the C═O stretching peak exhibited a similar shift to 1737 cm\(^{-1}\). The shifts observed in the peaks corresponding to the carbonyl groups may be attributable to Span–cholesterol interactions, namely hydrogen bonding, an interaction characteristic of the formation of niosomes.

In the spectrum of the drug-loaded niosomes, notable peaks were observed at 3370, 2920, and 1738 cm\(^{-1}\). The peaks likely correspond to the OH stretching, carbonyl dimer, and C═O stretching respectively. Similar to what was seen in the spectrum of the blank niosomes, significant shifts are observed in the carbonyl dimer and C═O stretching, which are indicative of the interactions mediating the niosomal formation. The shift seen in the OH stretching peak of AZM suggests that the likely mechanism by which AZM is being incorporated in the niosome is hydrogen bonding, which suggests that AZM is being encapsulated within the inner

### Table 5. Zone of Inhibitions (mm) of Tested Formulation Against S. aureus and S. epidermidis.

| bacterial strain   | NC | Well (2) | Well (4) | Well (3) |
|-------------------|----|----------|----------|----------|
| Staphylococcus aureus | 0  | 2.8      | 2.6      | 2.5      |
| Staphylococcus epidermidis | 0  | 2.6      | 2.2      | 2.0      |

*Well no.1: negative control (NC) (1% ethanol + PBS), well no.2 and 4: positive control (PC) (azithromycin 15 μg/100 μL), and well no.3: test item 15 μg/100 μL.*

*Figure 5. Zones of inhibition of (A) Staphylococcus aureus and (B) Staphylococcus epidermidis using the niosomal formulation (F2). Note: scale: 1 mm. Well no.1: NC (1%ethanol + PBS), well no.2 and 4: PC (AZM 15 μg/100 μL), and well no.3: test item 15 μg/100 μL.*

*Figure 6. FTIR spectra of the raw materials, unloaded niosomes, and drug-loaded niosomes.*
aqueous compartment of the niosome, a hypothesis which is not unlikely due to the hydrophilic nature of the drug.

### 3.7 Characterization of Niosomal Gels

Based on the characterization results of niosomes that have been attained, F2 showed the highest percentage of the drug entrapped, release, and stable system; it was then selected to be loaded into a gel formulation. Niosomal gels were successfully prepared using different polymers such as Carbopol and Na-CMC. The appearance, pH, viscosity, and ex vivo drug release of niosomal gel formulations were examined. The consistency and homogeneity of the gel were tested by pressing a tiny amount of it between the thumb and the index finger. The gel formulation had no coarse particles. The appearance of the niosomal gels was found to be translucent white in color. The pH of the gel was 6.56 ± 0.05, within the neutral normal range of pH for topical preparations, indicating that it can prevent any potential skin irritation.

### 3.8 Rheological Studies

#### 3.8.1 Viscosity Measurements

The AZM-loaded niosomal gels (NG-1 and NG-2) and AZM-loaded conventional gels (CG-1 and CG-2) exhibited a pseudoplastic (thinning) flow with high viscosity at low shear rates and low viscosity at high shear rates (Figure 7).

The flow curves showed that NG-1 and NG-2 displayed lower viscosity values than their corresponding conventional gels (CG-1 and CG-2). The lower viscosity of NG-1 and NG-2 might be attributed to the addition of niosomes to gels, which might interfere with the Carbopol and Na-CMC gel networks, promoting a weakening of the interactions between the interpolymer connections and hence reducing their thickening efficiency. This is in agreement with Manosroi et al. and Kumbhar et al., who reported that the incorporation of niosomes into Carbopol gels markedly decreased their viscosity. In addition, the viscosity of CG-2 was higher than that of CG-1, where the combination of Carbopol and Na-CMC in CG-2 led to viscosity synergism.

The viscosity data of NG-1, NG-2, CG-1, and CG-2 gels were fitted into the Carreau-Yasuda model with a correlation coefficient ($R^2$) of 0.9348–0.9904 and Casson models with $R^2$ of 0.9798–0.9940. The calculated zero-shear rate viscosity ($\eta_0$), infinite viscosity at zero shear rate ($\eta_\infty$), yield stress ($\tau_0$), and $R^2$ values are summarized in Table 6. The yield stress ($\tau_0$) represents the initial resistance for the gels to flow when the samples are exposed to external shear deformation, where gels do not flow unless the applied stress exceeds this yield stress value. Yield stress is a response parameter that reflects the gel strength and rigidity. All gels exhibited yield stress values, indicating a shear-thinning flow. CG-2 exhibited the highest yield stress of 16.80 Pa in agreement with the viscosity data.

#### 3.8.2 Viscoelastic Properties

The LVE region of low strain values was determined during the strain-sweep experiments. In this region, gels remained unbroken over the strain range. The LVE region of the E∗′ vs γ curve is marked by a point known as a critical strain ($\gamma_c$) where $G^\prime$ and $G^\prime\prime$ change as a function of strain, and the deformation of gels becomes irreversible. The LVE regions and critical strains of each gel are illustrated in Table 7. A constant strain value of 0.1% was selected for all gels to run the frequency-sweep measurements and determine the viscoelastic properties of the gels. These strain values were located within the LVE region of each gel. The viscoelastic properties are a distinctive characteristic that describes the relative contributions of the elastic and viscous behavior of gels and hence plays a major role in imparting spreading properties, retention time at the site of action, and drug release rate.

Table 7: VLE Region and Critical Strain ($\gamma_c$) of CG-1, CG-2, NG-1, and NG-2 Gels Determined From the Strain-Sweep Experiments

| gels | LVE region | $\gamma_c$ (%) |
|------|------------|----------------|
| CG-1 | 0.01–2.5   | 2.5            |
| CG-2 | 0.01–0.25  | 0.25           |
| NG-1 | 0.01–0.39  | 0.39           |
| NG-2 | 0.01–2.5   | 2.5            |

Figure 8 represents $G^\prime$ and $G^\prime\prime$ frequency-sweep curves of NG-1, NG-2, CG-1, and CG-2 gels. The four gels exhibited a domination of the elastic modulus ($G^\prime$) over the viscous modulus ($G^\prime\prime$) in the investigated frequency range (0.1–100 rad/s), indicating stable gels with more solid-like properties. The CG-2 gel exhibited higher values of $G^\prime$ and $G^\prime\prime$ than those of CG-1, suggesting a more stable and rigid gel structure for CG-2. This finding is in accordance with the viscosity data (Figure 7).

The addition of niosomes preserved the viscoelastic properties of gels with $G^\prime>G^\prime\prime$. Nevertheless, the influence of niosomes on the viscoelastic properties of NG-1 and NG-2 gels varied based on the composition of gels. For instance, niosomes were able to strengthen the gel network of NG-1, as evidenced by the higher $G^\prime$ and $G^\prime\prime$ values compared to those of its corresponding gel (CG-1) (Figure 6A).
finding indicates that NG-1 became more elastic, thus withstanding the stress resulting from the application on the skin. In contrast, niosomes dramatically decreased the viscoelastic properties of NG-2 gel, as evidenced by $G'$ and $G''$ values, which were lower than those of its corresponding gel (CG-2) (Figure 6B). This finding is in accordance with viscosity data, which showed that the addition of niosomes resulted in a reduction in the viscosity of NG-2 gel. Although the viscoelastic properties of niosomal gels either increased (as in NG-10) or decreased (as in NG-2) upon the addition of niosomes, the inclusion of AZM niosomes did not change the rheological response where a domination of the elastic modulus was found in the niosomal gels. It has been reported that the impact of niosomes on the rheological properties of gels might vary based on the composition of niosomes and the interactions between gel-forming polymer and niosomal lipids, which can affect the gel consistency and hence its rheological behavior.  

3.9. Ex Vivo Study. AZM is a BCS class III drug; therefore, nanocarrier formulations have been developed to enhance its permeation. One of the most promising passive methods developed to penetrate the SC is the use of vesicular systems, particularly niosomes. Niosomes were found to be capable of increasing the drug’s residence time in the epidermis, which could potentially enhance its permeation. In order to understand the ability of the niosomal gel to aid AZM permeation through the skin, ex vivo studies were conducted for niosomal formulations (NG-1 and NG-2) and compared with the conventional drug-loaded gels (CG-1 and CG-2) to present better comparisons of the permeation behavior through the rat skin as shown in Figure 9. AZM was not detected in the receptor compartment until 24 h of investigation in any of the conventional gels (CG-1 and CG-2), where the drug content was below the limit of detection. In contrast, the AZM niosomal gels penetrated the skin as early as the first hour of evaluation (Figure 9). It was observed that conventional gels showed a very low release percentage at 24 h: 1.59 ± 0.20 and 1.25 ± 0.12% for CG-1 and CG-2, respectively, which was significantly ($P < 0.0001$) lower than that for the niosomal gels. NG-2 niosomal gel showed 90.83 ± 3.19% AZM, whereas NG-1 niosomal gel had only 39.67 ± 7.53% AZM after 24 h. Thus, the NG-2 formulation showed a twofold increase in diffusion as compared to the NG-1 niosomal gel. In addition, it is obvious from the results that niosomal gels have a significantly greater release in comparison to conventional gels. This finding is in agreement with a
previous study, where Patel et al.\textsuperscript{60} developed a niosomal gel for improving the systemic availability of lopinavir and reported a significantly higher extent of absorption of lopinavir via transdermally applied niosomal gel as compared with its conventional dosage form (oral suspension). Meloxicam niosomes have also been prepared using Span 60 and cholesterol at different molar ratios for transdermal use after their incorporation into three different gel bases. Data reported by El-Badry et al. showed superior drug release from niosomal gel when compared with the conventional gel formulation.\textsuperscript{61}

The AZM release from niosomal gel was extended for 24 h. An ideal extended-release delivery system must provide a low initial release, but most of it should be released in a controlled manner over a defined period.\textsuperscript{62} It was obvious that the niosomal gel increased AZM permeation and allowed for longer release. This demonstrated that the prepared niosomes had a controllable release characteristic. AZM was released from niosomal gels through a combination of drug release from niosomes and then diffusion across the gel’s polymer network.\textsuperscript{63} The AZM could gradually leach out of the vesicles and into the surrounding gels. The surfactant Span 60 was thought to be a component that may increase skin permeability in many previous studies\textsuperscript{39} since the presence of a surfactant in the structure of niosomes aids in the solubilization of lipid in the SC, allowing for greater vesicle penetration.\textsuperscript{64} Because of their hydrophilic nature and bioadhesive qualities, Carbopol and Na-CMC polymers were chosen to prepare the niosomal gels in this study, which may result in an enhanced residence period of a drug at the absorption site through interacting with SC.\textsuperscript{65,66} The increased drug skin retention in the case of niosomal gels could be attributed to the drug creating a reservoir effect in the skin and boosting drug absorption in the skin. Niosomes have been shown to improve drug permeation through the skin.\textsuperscript{37,66} Niosomes modify the properties of SC by adsorption and fusion of niosomes onto the skin’s surface, which would facilitate drug penetration.\textsuperscript{66,67} In addition, structure modification of the SC is one of the proposed mechanisms for niosomal augmentation of drug permeability.\textsuperscript{68} Moreover, the viscosity of the gel formulation influences drug release significantly because it influences the rate of drug diffusion from the carriers.\textsuperscript{68} The flow curves showed that NG-1 and NG-2 displayed lower viscosity values than their corresponding conventional gels (CG-1 and CG-2). Several studies have indicated that increasing viscosity has a deleterious impact on drug release and skin penetration in vitro.\textsuperscript{68–70} As a result, gels having a higher viscosity released drugs at a slower rate.

4. CONCLUSIONS

AZM-loaded niosomes offered an alternative approach to deliver AZM in patients using a stable, low-cost method. In the current study, an attempt was made to develop a niosomal gel for improved systemic availability of AZM via a transdermal route. As suggested by the results, a niosomal formulation (F2) with the highest percentage of drug entrapped, the best release percentage, and the best stability results was chosen for gel formulation. The niosomal gel loaded with AZM showed greater release and prolonged action than conventional gel formulations containing AZM in the non-niosomal form. Overall, these results indicate that the niosomal gel could be an effective transdermal nanocarrier for controlling and enhancing the permeability of AZM, a BCS class III drug. In conclusion, this study suggests that transdermal formulations of AZM in niosomal gel were successfully developed and could be used as an alternative route of administration.

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Notes

The authors declare no competing financial interest.

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