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Original Article

Preparation, characterization and toxicological evaluation of azithromycin-loaded chitosan nanoparticles alone and in combination with cetirizine dihydrochloride

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Abstract

Respiratory tract infections are becoming difficult to treat due to multidrug resistance (MDR) bacteria. Nanoparticles (NPs) are suitable substitutes to circumvent MDR. This study was designed to formulate, characterize, and investigate the safety evaluation of azithromycin-loaded chitosan nanoparticles (AZM-CSNPs). AZM-CSNPs were prepared using the ionic gelation method and were characterized by Scanning Electron Microscopy (SEM), Fourier Transform Infrared Spectroscopy (FT-IR), zeta potential, entrapment efficiency, and *in vitro* drug release. Genotoxic and cytotoxic activity was determined by COMET and MTT assays. The practical yield of NPs was 77%. FT-IR illustrated the peak appearance at 1638 cm^{-1}, representing the formation of NPs. The bending of spectrum at 1525 cm^{-1} corresponds to chemical cross-linking with polymer attributing C-N bonds. The average size of nanoparticles was 64 nm with a zeta potential of +26.5mV and polydispersity index of 0.214, which expresses good stability. SEM image exhibited nearly spherical-shaped NPs owning smooth surfaces with entrapment efficiency of 71.14%. Chitosan nanoparticles bestow maximum drug release at acidic pH. The general release profile of the drug was divided into two basic phases: 10% initial burst release at 10hrs then a gradual release after 24hrs. Furthermore, the outcome elucidates that AZM-CSNPs do not cause DNA damage and there was no cytotoxic effect observed on Vero cell lines. Our results revealed that the combination of AZM-CSNPs with cetirizine dihydrochloride may be considered an innovative and promising strategy to improve the efficacy and targeted drug delivery and thus could be an effective approach to prevail over azithromycin resistance.

Keywords: Azithromycin, Chitosan nanoparticles, MTT, COMET, Cetirizine dihydrochloride, Azithromycin resistance

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Introduction

Respiratory tract infections (RTIs) are one of the major causes of morbidity and mortality (de Steenhuijsen Piters et al., 2020). Chemicals known as antibiotics are hazardous to cells in various ways. Antibiotic concentration that is administered to the body is frequently a determinant of their toxic effect on healthy eukaryotic cells (Ahsan et al., 2024). Antibiotics are being overprescribed for upper respiratory tract infections, which give rise to problems associated with resistance (Fülöpová et al., 2021). Correct identification of pathogens and their antibiotic sensitivity testing are crucial parameters for selecting suitable and successful antibiotic therapy in lower respiratory tract infection and thus helpful in preventing antibiotic resistance (Santella et al., 2021). Respiratory tract infections are major infections that require treatment (Kollef et al., 2021). Today, one of the substantial ultimatums to public health is MDR bacteria. Several alternative therapies for the treatment of MDR bacteria have drawn greater interest in developing newer conventional antimicrobial agents to enhance therapeutic efficacy (Iszatt et al., 2021; Rasool et al., 2024).

Azithromycin is an antibiotic derived from erythromycin with a wider spectrum of activity and better absorption (Finberg and Guharoy, 2021). Azithromycin has demonstrated therapeutic benefits in individuals with persistent respiratory tract infections (Beckert et al., 2021). Gram-positive bacteria that are resistant to macrolides usually do so due to changes in the target site or possibly by an active efflux pump mechanism. Gram-negative bacteria, on the other hand, are thought to be naturally resistant to macrolides due to the higher permeability of their cell membrane to hydrophobic compounds and the high efficacy of their efflux pump system (Meerwein et al., 2020). Macrolide-resistant *Staphylococcus aureus* (MAC-*MRSA*) is a major therapeutically admissible pathogen because of its limited treatment of choice and has high potential to develop resistance to various antimicrobials (Bishr et al., 2021). *Klebsiella pneumoniae* (*K. pnumoniae*) is a member of the Enterobacteriaceae family and was found in the number of samples collected from hospitalized individuals having respiratory problems (Asghari et al., 2021).

Different non-antibiotic medications, such as antihistamines, showed bactericidal action against various tested isolates (Boyd et al., 2021). The presence of aromatic rings in the molecular structure of antihistaminic drugs increases their antimicrobial action. Repositioning several non-antibiotic classes as appealing options for treating severe bacterial infections brought on by multidrug-resistant (MDR) bacteria that are significantly resistant to antibiotic therapy alone (Foletto et al., 2021; Rasool et al., 2024). Cetirizine dihydrochloride's antibacterial action revealed bacteriostatic activity against various Gram-positive and Gram-negative tested microorganisms, suggesting that it has potential to be an antibacterial agent in the future (Lagadinou et al., 2020).

Chitosan is frequently used biopolymer due to the variety of its physicochemical properties, such as biocompatibility, biodegradability, and non-toxicity, which is a promising approach for biomedical applications, chitosan-based nanoparticles are at the vanguard and spark a lot of interest. It is well known that chitosan- nanoparticles exhibited antibacterial action against a wide range of infections. As a result, they are now a highly pertinent candidate to fight against this wave of resistance (Alqahtani et al., 2020). Physical reciprocation by ionic gelation method was discovered to be the most efficient method among all the methods used for the preparation of nanoparticles. The development of electrostatic interaction between cationic group of positive charge bearing chitosan (CS) and the anionic negatively charged group of tripolyphosphate (TPP) is taken advantage of by the ionic gelation process. The size and surface charge of NPs might be altered by diversifying the ratio of (CS) to TPP in Nano formulation (Chandrasekaran et al., 2020; Afzal et al., 2024; Mahmood et al., 2024). Azithromycin-loaded chitosan nanoparticles demonstrated a wider zone of inhibition and greater antibacterial efficacy against *Escherichia coli* (Srag El-Din, 2022). It has been discovered that drug containing nanoparticles enhance solubility, bioavailability, stability, pharmacokinetics, and drug targeting. Thus using nanotechnology can effectively overcome resistance (Abo-zeid et al., 2021). The RTIs are becoming more difficult to treat due to the irrational use of antibiotics. Azithromycin resistance is posing serious health issues. The antibacterial activity of cetirizine dihydrochloride has been disclosed against various Gram-negative and Gram-positive bacteria. Nanoparticles, as one of the most recent innovative drug delivery carriers, have been proven to improve drug efficiency by targeting drug delivery and increasing bioavailability. Keeping

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in view the nanoparticles were formulated by loading the drug on chitosan to combat azithromycin resistance in respiratory tract infections caused by resistant isolates of *K. pneumoniae* and *MRSA*. The current study's objectives are to develop, characterize, and assess the safety of AZM-CSNPs. This combination may be safer and helpful to minimize azithromycin resistance.

Material and Methods

Chemicals and reagents

Azithromycin dihydrate USP (Hebei Dongfeng pharmaceutical Co. LTD:B#A20201127), Cetirizine dihydrochloride BP (Sreekara Organics, ISO 9001: 2015 certified company B#CTZ05719), Low Molecular Weight chitosan (50−190 kDa: B#STBH 6262) , Sodium tripolyphosphate (MW 367.86 g/mole) , Phosphate buffer saline, 1% v/v acetic acid, Tween 80, Hydrochloric acid, Dialysis membrane (MWCO 3500Da), MTT dye, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (0.25%), Dimethyl Sulfoxide (DMSO10%), Dulbecco's modified Eagle medium (DMEM) and RPMI1640 medium, Disodium ethylene diamine tetra acetic acid (disodium EDTA), Trizma base, sodium dodecyl sulfate, triton X 100, and lymphocytes separating medium. All materials were of analytical grade, procured from Sigma Aldrich Chemical Co. (St. Louis, MO).

Preparation of Azithromycin-loaded chitosan Nanoparticles (Ionic Gelation Method)

Chitosan solution was obtained by dissolving chitosan powder in 1% v/v aqueous acetic acid solution. Tween 80 was added as a surfactant and then agitated at 60°C for 2 hrs. The pH of the solution was adjusted to 4.4. *For AZM-CSNPs,* azithromycin was dissolved in CS solution (1:1 w/w). This solution was mixed with the stirring mixture at a rate of 1ml/min in a weight ratio of 1:1 for 25 minutes with the addition of tripolyphosphate. Nanoparticles were collected and centrifuged at 9000 rpm for 20 minutes. The lyophilized nanoparticles were attained as fine powder and were then stored at 4°C in a refrigerator until used in the research project (Srag El-Din, 2022).

Practical yield of nanoparticles

The lyophilized nanoparticles were weighed to measure the percentage yield; it was calculated from the weight of freeze-dried nanoparticles dried from all the batches. The recovered nanoparticles were weighed and divided by the sum of the initial material used. The percentage yield was calculated by using the following equation (Divya et al., 2017).

$$\textbf{\textit{Practical yield}}\ \% = \frac{\textbf{nanoparticles weight}}{\textbf{\textit{theoratical mass}}} \times \mathbf{100}$$

Evaluation of entrapment efficiency of Azithromycin-loaded chitosan nanoparticles

A sample of 50 µl was collected from 50 ml of nanoparticle stock solution and centrifuged at 8000 rpm at 4 ºC for 10 minutes. The resilient containing non-entrapped drug was retained. A standard calibration curve was obtained by using different concentrations (20, 40, 60, 80, 100µg) at 216 nm by using a UV-Vis spectrophotometer (UV-2001 Hitachi, Tokyo, Japan).

The entrapment efficiency (EE) of AZM-CSNPs was calculated by following the equation (Gooneh-Farahani et al., 2020).

$$\textbf{\textit{Drug entrapment}}\ (\%) = \frac{\textbf{Total drug–Free drug}}{\textbf{Total drug}} \times \mathbf{100}$$

In vitro drug release profile

A relative *in vitro* drug release study was done in phosphate buffer solution (PBS), having three different pH values, acidic 4, neutral 7.4, and basic 10. A weighed amount of nanoparticles was disseminated in 3ml of PBS and transferred into the dialysis membrane. The dialysis membrane sealed at both ends, was placed into the beaker containing 100 ml of PBS. To quantify the concentration of the drug in the receiving compartment, samples (5ml) were taken out of the beaker at regular intervals of 0, 1,4,6,24,48 hr (Bekele and Alamnie, 2022). The percentage of azithromycin released at each point at a specific interval of time was determined by using the following equation.

Drug release from nanoparticles (%) = Released drug at a desired time / Total amount of drug entrapped within nanoparticles x 100 %

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Characterization of nanoparticles

Fourier Transform Infrared Spectroscopy analysis (FT-IR)

To determine the chemical interaction between chitosan and TPP, Fourier transforms infrared (FT-IR) spectra were used with a spectral resolution of 4 cm^{-1}, and sample spectra were captured in the middle infrared band between 400 and 4000 cm^{-1}. Potassium bromide (KBr) was combined with chitosan powder and chitosan nanoparticles before being pressed into pellet form (Benamer Oudih et al., 2023).

Scanning Electron Microscopy (SEM)

Scanning electron microscopy (JEOL: JSM.6480LV) was used to examine the shape and surface morphology of the chitosan NPs. Before analysis, the sample was mounted on carbon tape and coated with gold for one minute under vacuum (Waqas et al., 2022).

Determination of particle size and Zeta potential of nanoparticle

To estimate the particle size (PS), the value of polydispersity index (PDI) and zeta potential (ZP) was obtained through Zeta sizer (Malvern Instruments, U.K.), in which a suitable dilution of the nanoparticle's dispersion was prepared utilizing dynamic light scattering technique. At a temperature of 25 °C and at 90° angle, the `dispersion was observed (Kandav et al., 2019).

Toxicological evaluation of Azithromycin-loaded chitosan nanoparticles

Single Cell Gel Electrophoresis / Comet Assay

Azithromycin, cetirizine dihydrochloride, and AZM-CSNPs and their combinations were evaluated for genotoxic potential. The buffy layer containing sheep lymphocytes was obtained and combined in equal parts with RPMI-1640 medium and centrifuged at 800X for 15 minutes. The drugs were serially diluted by two-fold serial dilutions, concentrations ranging from 1000 to 31.25µg/ml and each dilution was then exposed to 100 µl of lymphocyte suspension. After incubation, 10 µl of the treated lymphocytes were combined with 65 µl of the low-melting agarose. Slides were treated with an alkaline buffer then placed in an electrophoresis tank for 30 minutes. The slides were stained with a neutralizing buffer solution for five minutes. Ethidium bromide stain was used for slide staining and was viewed at a magnification of 40X (Møller et al., 2020).

Comet scoring

COMET IV image software was used for comet scoring all slides coded according to their concentration.

Control group

Positive group

DMSO 20% (Dimethyl sulfoxide)

Negative group

Normal saline.

Comet scoring formula

Cells were divided into four groups according to their tail length.

Class 0 = undamaged cells, **Class 1**= tail length less than or equal to head diameter, **Class 2**= tail length is more the diameter of the head and less the double diameter of the head, **Class 3**= tail length greater than the double diameter of the nucleus head.

Damage index= Number of cells in Class 1 + (2 x Number of cells in Class 2) + (3x Number of cells in Class 3).

Genetic Damage index GDI= No. of cells in Class 1+(2 x No of the cells in Class 2) + (3 x No of cells in Class 3) / No of cells in Class 0 + No of cells in Class 1 + No of cells in Class 2 + No of cells in Class 3.

Based on the above-mentioned criteria genetic damage index and damage index were calculated as described by (Tung et al., 2023).

MTT assay (Cytotoxic evaluation)

By using an *in vitro* colorimetric MTT (dimethylthiazol diphenyl tetrazolium) assay, the cytotoxicity of prepared formulations was evaluated. Vero cell lines were used in the experiment. The University of Veterinary and Animal Sciences Lahore's Quality Operation Laboratory gathered and processed the cryopreserved vials containing Vero cell lines. The strength of the azithromycin, cetirizine dihydrochloride, AZM-CSNPs, and their combination for a stock solution was prepared 2000µg for each in different eppendorf tubes. Initial dilution of 100 µl of diluent was performed, followed by the addition of 100µl from the first well containing 1000 µg and incorporation into the second well, followed by two-fold serial dilutions and so on up to the tenth well. Following a 24-hour incubation period, 100µl from each well was removed and 100µl of MTT dye was added. The insoluble formazan was then dissolved by adding 100µl of 10% DMSO to each well and optical density measurements were made at 570 mm using an ELISA microplate reader (Ghasemi et al., 2021).

$$CSP = \frac{\text{Mean Optical Density of test} - \text{Mean Optical Density of negative control}}{\text{Mean Optical Density of positive control}} x\ 100$$

Inhibitory concentration IC_{50}

The IC_{50} values of azithromycin, cetirizine dihydrochloride, and AZM-CSNPs, and their combinations were calculated by plotting a graph between concentrations and samples of drugs and nanoparticles (Ilaghi et al., 2021).

Results

Practical yield of nanoparticles

After successful loading of azithromycin on chitosan nanoparticles the calculated practical yield of nanoparticles was 77%.

Drug Entrapment and *In Vitro* Drug Release Profile

The standard calibration curve of AZM-CSNPs is given in (figure-1), where the regression equation is represented by a constant line:

$y = 0.0007x + 0.028$, $R^2 = 0.9919$

where Y indicates the absorbance of nanoparticles at 200-400 nm and R^2 is the associated factor. By using this calibration curve, entrapment efficiency was calculated as 71.40%. Azithromycin released from AZM-CSNPs was continuously monitored for up to 48 hours at pH 4, 7.4, and pH 10, drug release was 67.5 % at pH 4 while 54.7% at 7.4 and it was 44.8% at 10 (figure-1). AZM-CSNPs showed extremely gradual release with an initial abrupt release. Nanoparticles provide maximum drug release at low or acidic pH as chitosan dissolves at lower pH. The general release profile of drug from NPs could be divided into two basic stages: 10% initial burst release at 10 hours, then a 24-hour gradual drug release.

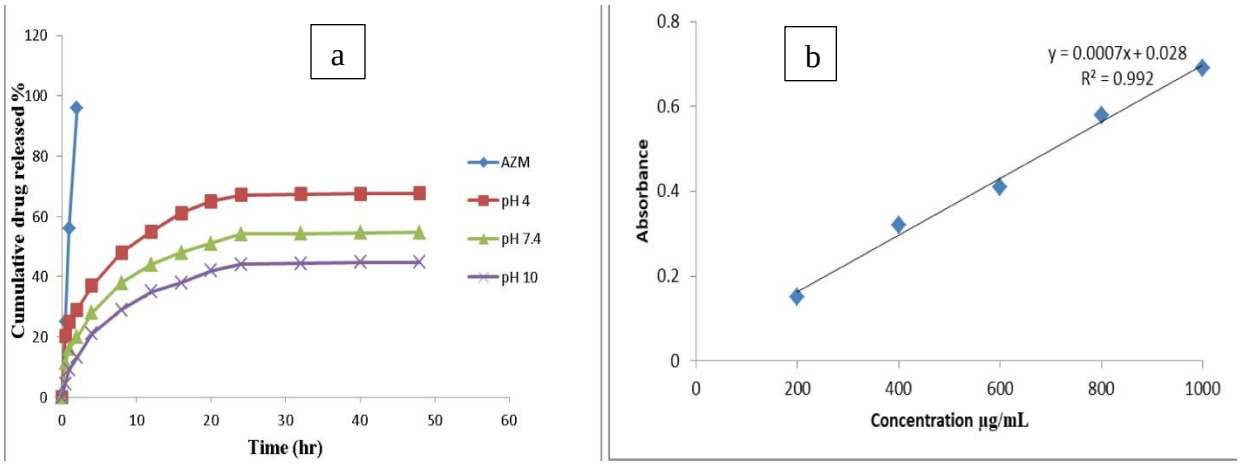

Figure-1 (a) Comparison of *In vitro* drug release at different pH (b) Standard Calibration Curve

Characterization of nanoparticles

FTIR spectra of Chitosan

FT-IR spectra of chitosan in our study represented a peak at 3453 cm^{-1} that corresponds to the OH hydroxyl group. Peak at wavelength of 2925 cm^{-1} represents stretching vibration which was a characteristic feature of pyranose ring in structure. The bending of the spectrum at 1658 cm^{-1} was associated with CO in the amide group while 1415 cm^{-1} and 1320 cm^{-1} represented vibration of OH, and CH bond in ring, respectively. The bending spectrum was observed at 1150-1040 cm^{-1} describing the presence of glycosidic bond and CH_3OH group was seen at wavelength of 850-838 cm^{-1}.

FTIR spectra of Azithromycin

The FTIR spectrum of azithromycin represented typical peaks for the carbon double bond that appeared in the azithromycin spectra at around 1750 cm^{-1}. Clear

peaks at approximately 1000-1300 cm^{-1} for C-O bond stretching represented the presence of ether. A sharp peak at approximately 3600 cm^{-1} corresponds to free O-H stretching that is a typical indicator of the presence of water in the crystal lattice. The peaks below 3000 cm^{-1} represented aliphatic C-H stretch**.** Similarly, the peak at 3490 cm^{-1} represented O-H stretching that showed the presence of bound water in the crystal lattice with strong intermolecular forces (hydrogen bonding). The peak at 2973 cm^{-1} corresponds to – C-H stretching while the appearance of the peak at 1720 cm^{-1} represented a carbon double bond (C=O) and peak at 1186 cm^{-1} showed R-O-R ether stretching,1080 cm^{-1} related to C-N bond, respectively.

FTIR spectra of azithromycin-loaded chitosan nanoparticles

The FTIR spectrum of AZM-CSNPs represented peaks at 3327- 3070 cm^{-1} related to –OH vibration, N-H stretching and enhanced hydrogen bonding. The peak at 2924 cm^{-1} was attributed to AZM-CSNPs. The appearance of a new peak at 1638 cm^{-1} represented the formation of nanoparticles. The bending of the spectrum at 1525 cm^{-1} corresponds to chemical cross-linking with polymer attributing C-N bonds (phosphate group of TPP and N-O amino group of chitosan, amino group bending vibration became sharper represented high degree of deacetylation of chitosan). Other bending at various wavelengths of 1425 cm^{-1} represented C-C in the ring, 1069 cm^{-1} showed symmetrical and symmetrical stretching of the PO_3 group, and the band observed at 894 cm^{-1} was representative of P-O-O Asymmetric stretching in Nano formulation.

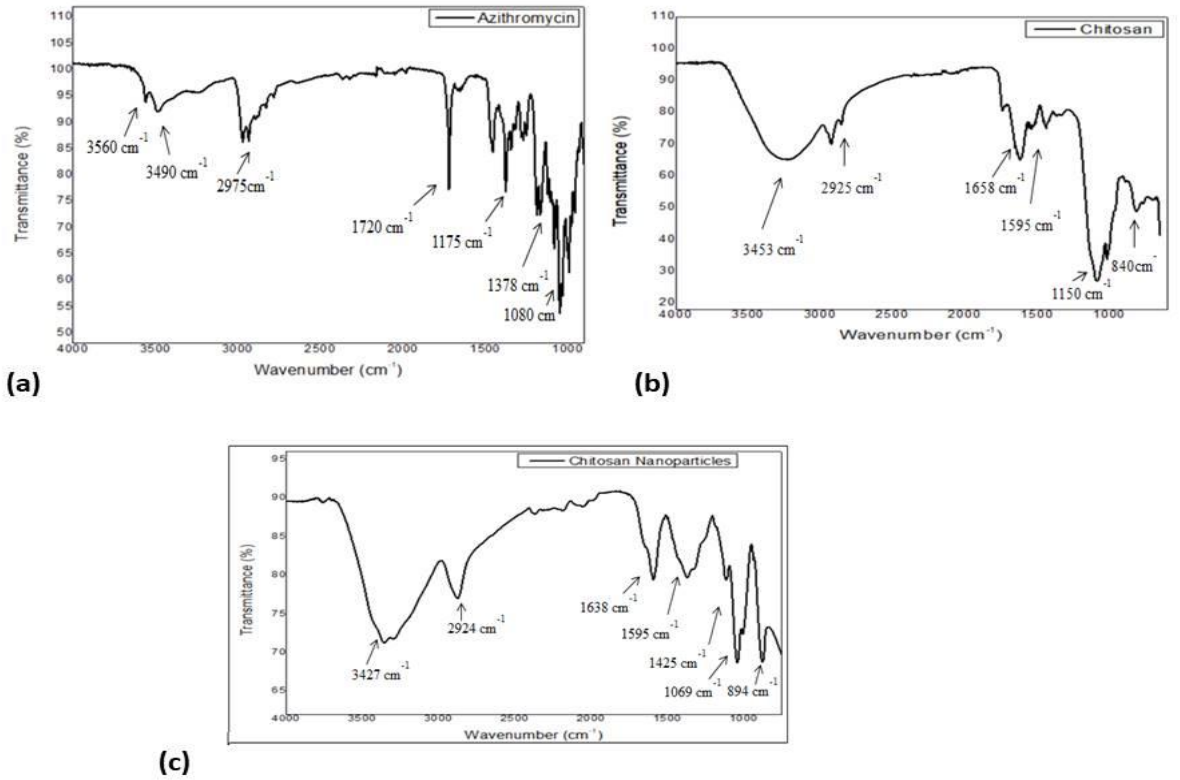

Figure-2 (a) FTIR spectra of Azithromycin (b) FTIR spectra of Chitosan and (c) FTIR spectra of azithromycin loaded chitosan nanoparticles.

Scanning Electron Microscopy

The SEM image of AZM-CSNPs showed nearly spherical shaped nanoparticles representing smooth surface. SEM micrograph of nanoparticles is given in (figure-3).

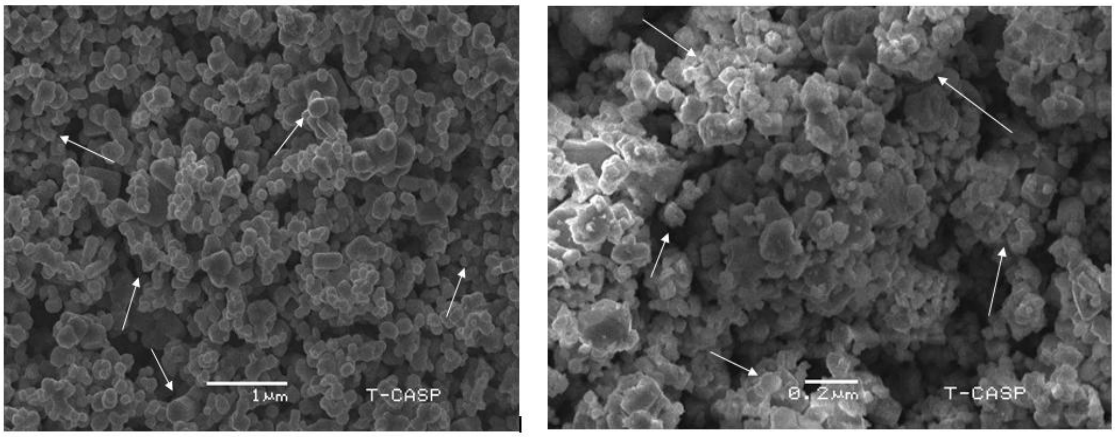

Figure-3 SEM micrograph for AZM-CSNPs

Zeta potential analysis

The size and charge of the nanoparticles were measured using a zeta sizer (Malvern, UK). The average size of the nanoparticles obtained after analysis was 64 nm, with a zeta potential of +26.5mV, which shows their good stability. The polydispersity index (PDI) was 0.214 (homogeneity).

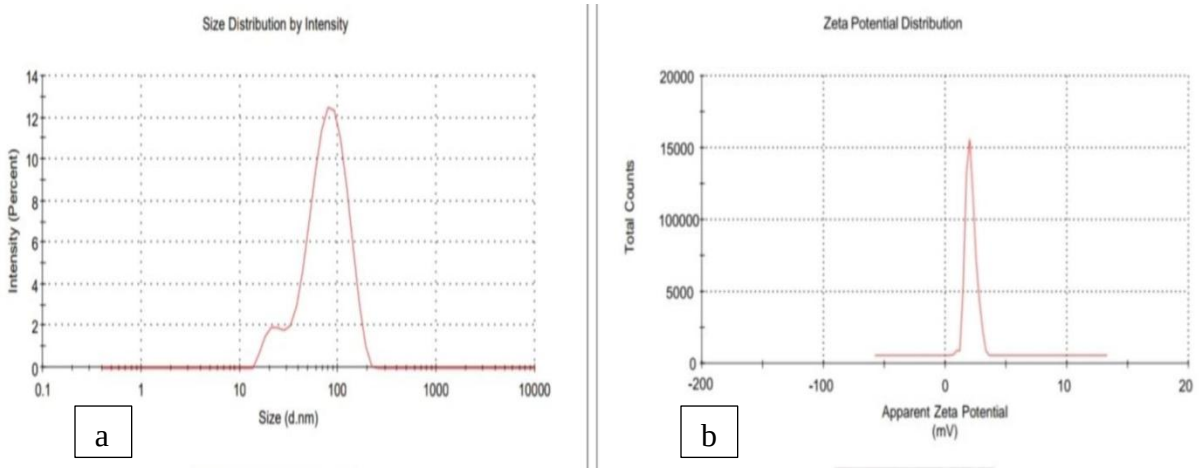

Figure-4 (a) Average size of AZM-CSNPs (b) Zeta potential of AZM-CSNPs

COMET assay (Single Cell Gel Electrophoresis)

The genotoxicity of combinations of azithromycin and AZM-CSNPs with cetirizine dihydrochloride were investigated at concentrations ranging from 1000µg/ml to 31.25µg/ml. For each dose total number of cells under observation was n=25. The results showed that AZM-CSNPs caused genetic damage at high concentrations of 1000µg/ml and 500µg/ml with a genetic damage index (GDI) of 0.48 and 0.4, respectively. However, all other concentrations tested were found to be safe. The mean tail length of DNA in cells exposed to AZM-CSNPs at the high concentrations of 1000 µg/ml, 500µg/ml was 4.43±0.03 µm and 3.94±0.03 µm respectively which was much lower than the positive control (20% DMSO) where the tail length was 9.98±0.03 µm. The combination of azithromycin and cetirizine dihydrochloride had a GDI of 0.44 and 0.32 at high concentrations of 1000µg/ml and 500µg/ml, respectively while rest of concentrations found safe. The mean tail length of DNA in cells exposed to this combination was 7.29±0.09µm and 6.26±0.04 µm, respectively, which was less damaged compared to the positive control. The AZM-CSNPs and cetirizine dihydrochloride combination exhibited a GDI of 0.48

at high concentration 1000µg/ml while the rest of concentrations were found to be safe. The mean tail length of DNA by exposure of this combination was 3.87±0.02µm which was less damaged compared to positive control. As the concentration of drug increased there was an observed increase in GDI. Thus the combination of AZM-CSNPs was found least genotoxic even at higher concentration of 1000µg/ml as compare to drug given alone. However, drug concentration of 250µg/ml, 125µg/ml, 62.5µg/ml and 31.25µg/ml were found to have less genetic damage index thus the genotoxic potential was completely dose dependent.

Table-1 Mean head and tail length of damaged DNA for Azithromycin, AZM-CSNPs and their combination with Cetirizine dihydrochloride.

	Mean DNA head length (µm) after different concentrations of drug (n=25)						**Mean DNA tail length (µm) after different concentrations of drugs (n=25)**					
Drugs	**Concentrations (µg/ml)**						**Concentrations (µg/ml)**					
	31.25	**62.5**	**125**	**250**	**500**	**1000**	**31.25**	**62.5**	**125**	**250**	**500**	**1000**
Azithromycin	7.97± 0.02	7.41± 0.02	6.84± 0.04	5.78± 0.01	4.57± 0.03	3.86± 0.05	2.32± 0.03	3.23± 0.02	4.04± 0.04	5.15± 0.05	6.28± 0.04	7.53± 0.03
Cetirizine dihydrochloride	7.35± 0.04	6.72± 0.03	6.02± 0.02	5.21± 0.01	4.13± 0.02	3.87± 0.02	6.19± 0.01	5.32± 0.03	4.01± 0.01	3.45± 0.04	2.93± 0.04	2.50± 0.01
Azithromycin + Cetirizine dihydrochloride	7.11± 0.02	6.21± 0.02	5.73± 0.03	5.01± 0.04	4.65± 0.05	3.72± 0.04	3.44± 0.04	4.05± 0.04	4.81± 0.02	5.40± 0.02	6.26± 0.04	7.29± 0.09
AZM-CSNPs	7.17± 0.04	6.77± 0.03	6.37± 0.02	5.93± 0.06	5.37± 0.07	4.47± 0.04	2.05± 0.04	2.51± 0.02	3.00± 0.01	3.53± 0.02	3.94± 0.03	4.43± 0.03
AZM-CSNPs+ cetirizine dihydrochloride	9.24± 0.04	8.91± 0.01	8.61± 0.02	7.99± 0.01	7.88± 0.01	6.97± 0.02	0.84± 0.03	1.13± 0.02	1.73± 0.04	2.07± 0.10	2.46± 0.05	3.87± 0.02
Positive control	1.45±0.02						9.95±0.01					
Negative control	9.91±0.01						0.29±0.01					

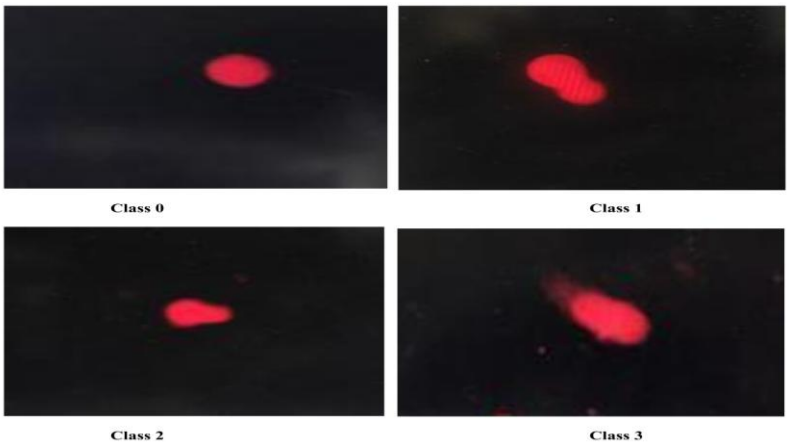

Figure 5. Class 0 (undamaged cell), Class 1 (tail length less than or equal to head diameter), Class 2 (tail length more than head diameter), and Class 3 (tail than greater than the double diameter of head).

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Evaluation of cytotoxicity by MTT assay

In sterile 96 well plates, two-fold serial dilutions of azithromycin, cetirizine dihydrochloride, AZM-CSNPs alone, and in a combination of azithromycin + cetirizine dihydrochloride and AZM-CSNPs + cetirizine dihydrochloride (1000, 500, 250, 125, 62.5, 31.25, 15.62, 7.812, 3.9 and 1.95µg/ml) were prepared and distributed to determine the cytotoxicity. The cytotoxic effect was dose-dependent, but no cell damage was observed for the dose-producing antibacterial effect. The concentration of drug that is required to decrease cell viability by 50% (IC_{50}) was calculated. It was revealed that the combination of the drug concentrations was not cytotoxic to the Vero cell line at the dose producing an antibacterial effect. The values of IC_{50} (µg/ml) of individual drugs and their combinations on Vero cell lines are summarized in the following (Figure 6).

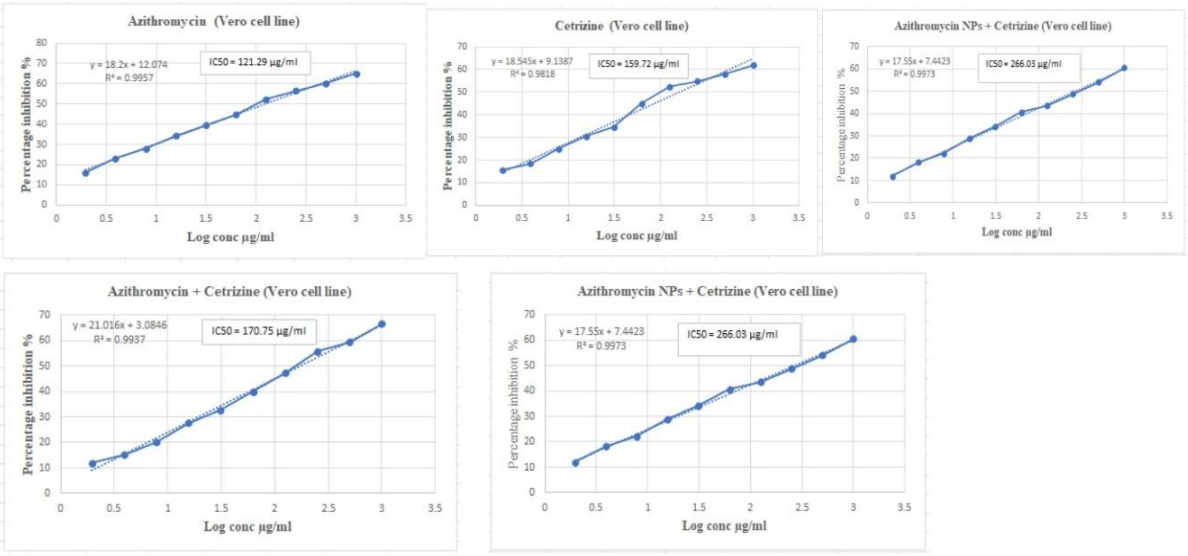

Figure 6. IC_{50} of drugs alone and in combination with cetirizine dihydrochloride

Discussion

The worldwide issue of antimicrobial resistance (AMR) is linked to elevated rates of mortality (Tran et al., 2023). In clinical respiratory isolates, resistance to macrolides is developing very fast and is becoming more prevalent (Yang et al., 2024). Combining evolutionary theory with nanotechnology presents promising approaches to addressing antibiotic resistance (Muteeb, 2023). Many people have recently become aware of chitosan because of its low toxicity, biocompatibility, and biodegradability (Wu et al., 2023). In our study, chitosan polymer was used for the preparation of AZM-CSNPs. Chitosan was used as an encapsulating agent to encapsulate azithromycin, so, AZM-CSNPs were obtained to improve the therapeutic activity of azithromycin and to enhance its antibacterial effect against *MRSA* and *K. pneumoniae*. The synthesized AZM-CSNPs were measured for particle size and zeta potential, and the findings showed that they were 276.5 nm and + 25 mV, respectively. The resultant encapsulation efficiency was 80% (Srag El-Din, 2022). The obtained result of zeta potential in our study was approximately the same as reported in above mentioned study; the measured charge on AZM-CSNPs was recorded as +26.5 mV. Depending on the type of drug being encapsulated on chitosan nanoparticles, entrapment efficiency of 70–75% has also been reported. Another study showed a high drug encapsulation efficiency of 82.94% (Chhonker et al., 2015). Similarly, a study conducted by (Kirimlioğlu and Öztürk, 2020) provided the information about the entrapment efficiency of 71.37 % which is the percentage of the amount of the drug loaded into polymeric matrices. In our study entrapment efficiency of was AZM-CSNPs calculated by using a calibration curve and it was 71.40% which is comparable with above reported studies.

The two-step *in vitro* drug release profile was explained by (Taghe et al., 2021). The amount of azithromycin released from the freeze-dried nanoparticles and free-form azithromycin during 48 hours were measured using a dialyzing bag approach.

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The entire drug release profile showed a burst release of the drug at first, which was followed by a persistent release of the medication via the nanoparticles. Similarly, in our research project azithromycin released from AZM-CSNPs was continuously monitored for up to 48 hrs at different pH. AZM-CSNPs showed extremely gradual drug release with initial burst release.

According to (Hasheminejad et al., 2019) chitosan nanoparticles exhibited a spherical form and smooth surfaces both when they were loaded and unloaded, the morphology of chitosan nanoparticles (CSNPs) was found to be quite uniform and spherical shape. The SEM image of AZM-CSNPs in this study showed nearly spherical shape nanoparticles with smooth surfaces having monodispersity in a formulation that represented similarity with another study in which the author reported the spherical shape and nanostructure of CSNPs, confirmed by SEM micrographs (Yilmaz et al., 2019).

The FT-IR spectra can be used to investigate the functional groups that are present in the Nano formulation. In the FT-IR spectra of CSNPs, the O-H stretching vibration in chitosan is frequently discernible as a peak at 3400 cm^{-1}(Divya et al., 2017). Our study also revealed a peak at 3490 cm^{-1} represented O-H stretching that showed the presence of bound water in the crystal lattice with strong hydrogen bonding. The bending of the spectrum was observed at 1150–1040cm^{-1} describing the presence of glycosidic interaction and the CH_3COH group was seen at a wavelength of 850 - 838 cm^{-1}.

A study reported by (Agarwal et al., 2018) revealed that chitosan nanoparticles showed a visible peak of the PO_4^{-2} group in the TPP spectra between wavelength range of 1138 $cm^{-1}$and 888 cm^{-1}representing the formation of nanoparticles**.** In our study, different peaks were observed that have been reported by (Hadidi et al., 2020) including 3327- 3070 cm^{-1} related to –OH vibration, N-H stretching and enhanced hydrogen bonding. The peak at 2924 cm^{-1} was attributed to azithromycin-loaded chitosan nanoparticles. The appearance of a new peak at 1638 cm^{-1} represented the formation of nanoparticles. The PO_4^{-2} group of TPP, and the N-O amino group of chitosan cause the bending of the spectrum at 1525 cm^{-1}. The sharpening of the amine group bending vibration indicates a high degree of acetylation of the chitosan. A band observed at 894 cm^{-1} was indicative of P-O-O asymmetric stretching in Nano formulation, while other bending at different wavelengths of 1425 cm^{-1} represented C-C in the ring while1069 cm^{-1} demonstrated symmetric and symmetric stretching of the PO_3^{-2} group.

Comet assay is a very helpful diagnostic tool for the determination DNA damage accurately and detection of genotoxicity of nanoparticles used in drug delivery systems (Vandghanooni and Eskandani, 2011). In our study, the genotoxic potential of azithromycin, cetirizine dihydrochloride, AZM-CSNPs, and their combinations were evaluated which represented the damage at a higher dose and there was no damage was observed at 125µg/ml, 62.5µg/ml, and 31.25µg/ml. Thus, the genotoxic potential was considerably dose-dependent. No previous studies have reported the genotoxic potential of nanoparticles. Our results suggested that the concentration of drugs alone and in combination represented a dose-dependent genotoxic effect and was not genotoxic at lower doses of 256µl to 31.25µl which is clear from changes in comet head and tail length diameter of DNA of sheep lymphocytes. The research findings are also similar to the study, in which human lymphocytes and V79 fibroblast cells were used to assess the cytotoxicity and genotoxicity profile of lecithin/chitosan nanoparticles with/without clobetasol-17-propionate (CP). Lecithin/chitosan nanoparticles with/without CP were found to be harmful at concentrations exceeding their IC_{50} values, although being at low concentrations. Lecithin/chitosan nanoparticles with/without CP have also appeared to cause genotoxicity at greater concentrations. In conclusion, nanoparticles can exhibit outstanding and practical properties for drug administration, but additional mechanistic research is required to ensure their safety (Taner et al., 2014). The cytotoxic effects of macrolides have been assessed before on human liver cell lines. Leukemic cells were used to assess the cytotoxicity of three semisynthetic macrolide antibiotics roxithromycin, clarithromycin, and azithromycin. The results showed that the cytotoxicity of macrolides was significantly dose-dependent (Tvrdá et al., 2016). Normal cells and cancer cell lines were tested. Most of the tests that are reported in this literature review were done on human cells and have shown little to no toxicity. Other forms of nanoparticles, such as chitosan nanoparticles, were not harmful to normal cells but exhibited cytotoxic effects on cancer cells. The cytotoxicity of nanoparticles can be affected by concentration, exposure time, and pH of solution (Zoe et al., 2023). The addition of chitosan

nanoparticles to the formulation resulted in greater antibacterial and less cytotoxicity compared with no addition of chitosan (Ratih et al., 2023).

Azithromycin dose-dependently reduced the growth of MCF-12A and fibroblast cells. Azithromycin's 50% inhibitory concentrations (IC_{50}) in MCF-12A and fibroblast cells were 94.33µg/ml and 115. 49µg/ml, respectively (Jiang et al., 2019). Our study also revealed that the cytotoxic effect was dose-dependent but there was no cell damage observed for the dose-producing antibacterial effect. Similar cytotoxic effect of silver nanoparticles, gold nanoparticles, and nano-formulated azithromycin was investigated and they did not cause any cytotoxicity in human peripheral blood cells (Namasivayam and Samrat, 2016). Our findings also agree with the reported study that there was no cytotoxic effect observed on Vero cell lines when azithromycin-loaded chitosan nanoparticles were given alone and in combination with cetirizine dihydrochloride. However, it was dose-dependent.

The growth inhibition of MCF-7, HeLa, and Vero cells with decreasing concentrations of single drug treatment (1000-1.95 µg/ml) was studied by measuring the mitochondrial activity of viable cells through MTT assay. The concentration required to decrease cell viability by 50% (IC_{50}) was determined using CompuSyn software. The IC_{50} values of drug combinations exhibited more pronounced dose-dependent growth inhibition of MCF-7 and HeLa cells as compared to drugs alone. It was revealed that none of the drugs were cytotoxic to the Vero cell line at any given concentrations (Akhlaq et al., 2023). Our findings also agree with the reported studies that there was no cytotoxic effect observed on Vero cell lines when AZM-CSNPs were given alone and in combination with cetirizine dihydrochloride.

Conclusion

The use of AZM-CSNPs as an adjuvant with cetirizine dihydrochloride can be a vital option to overcome resistance in respiratory tract infections, as it will enhance the stability and efficacy of the active compound as well as it will provide targeted drug delivery with reduced resistance and dose. The genotoxic and cytotoxic potential of AZM-CSNPs and their combination with cetirizine dihydrochloride was dose-dependent and found to be least toxic at lower doses thus proved safer and more effective than all the tested combinations.

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Contribution of Authors

Adeel Sattar, Muhammad Adil Rasheed & Muhammad Abu Bakr Shabbir: Designed the experiment. Umbreen Anwar & Adeel Sattar: Authored and summarized the results of experiments on the preparation and characterization of azithromycin-loaded chitosan nanoparticles.

M. Adil Rasheed & Umbreen Anwar: Summarized the results of COMET and MTT.

Mateen Abbas: Helped in the characterization of nanoparticles.

Umbreen Anwar: Collected data and compiled results.

All authors critically analyzed the results and approved the manuscript for submission.

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