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Azithromycin loaded chitosan nanoparticles preparation and in-vitro characterization

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Abstract

The aim of the current study was to prepare and evaluate the efficacy of novel Azithromycin loaded chitosan nanoparticles. Azithromycin loaded chitosan nanoparticles was prepared by ionic gelation of chitosan with tripolyphosphate anions. The prepared Azithromycin loaded chitosan nanoparticles was characterized by dynamic light scattering, zeta potential, encapsulation efficiency, in-vitro drug release for three hours, and in-vitro antibacterial activity against Escherichia coli. The results of particle size and zeta potential for the prepared Azithromycin loaded chitosan nanoparticles were determined and found to be 276.5 nm and + 25 mV respectively. The encapsulation efficiency result was 80%. The results after 3 hours of dissolution were 53.05 % for Azithromycin loaded chitosan nanoparticles and 7.25 % for azithromycin solution. Furthermore, the Azithromycin loaded chitosan nanoparticles showed broader inhibition zone and higher antibacterial activity against Escherichia coli compared with azithromycin solution. In conclusion, Azithromycin loaded chitosan nanoparticles are a promising platform for enhancing dissolution and antibacterial activity of Azithromycin against Escherichia coli.

Keywords: *azithromycin, chitosan, nanoparticles, ionic gelation*

Introduction

Macrolide antibiotics have been used in the treatment of infections caused by clinically important gram-positive cocci, such as Streptococcus spp. and Staphylococcus spp., and atypical pathogens, such as Mycoplasma spp., Chlamydia spp., and Legionella spp. In the 1990[s], the “new” macrolides, clarithromycin, and azithromycin were released. The new macrolides have an expanded spectrum of activity, including fastidious gram-negative bacilli, such as Haemophilus influenzae and Neisseria spp. (Imamura, Higashiyama, Tomono, Izumikawa, Yanagihara, Ohno, Miyazaki, Hirakata, Mizuta, Kadota, et al., 2005).

Azithromycin is a semi-synthetic antibiotic derived from erythromycin (Figure 1). It's an antibiotic called a Macrolide. This medication is an Azalide, a nitrogen-containing macrolide with indications and uses comparable to Erythromycin. By binding reversibly to the 50 S ribosomal subunits of sensitive bacteria, macrolides such as azithromycin suppress RNA-dependent protein synthesis (Imamura, Higashiyama, Tomono, Izumikawa, Yanagihara, Ohno, Miyazaki, Hirakata, Mizuta, & Kadota, 2005; Montejo-Bernardo et al., 2005). It is a di-cationic molecule that is distinguished by its capacity to penetrate the bacterial outer membrane, which is a crucial step in overcoming bacterial self-defence (Beckers et al., 1995; Imamura, Higashiyama, Tomono, Izumikawa, Yanagihara, Ohno, Miyazaki, Hirakata, Mizuta, & Kadota, 2005; Patil & Gaikwad, 2011).

Azithromycin is a broad-spectrum macrolide, an acid stable orally administered antimicrobial drug (Oliver & Hinks, 2021). It treats bronchitis, certain forms of skin infections, sore throats, pharyngitis, tonsillitis, nosocomial infections, and pneumonia when taken orally. Its one-of-a-kind trait is its outstanding action against Chlamydia trachomatis (Adeli, 2016). It is prescribed for the treatment of respiratory-tract infections (including otitis media) and also for skin and soft-tissue infections. Azithromycin can also be used for prophylaxis, and act as a component of drug regimen in the treatment of Mycobacterium avium complex (MAC) infections (Palanisamy & Khanam, 2011).

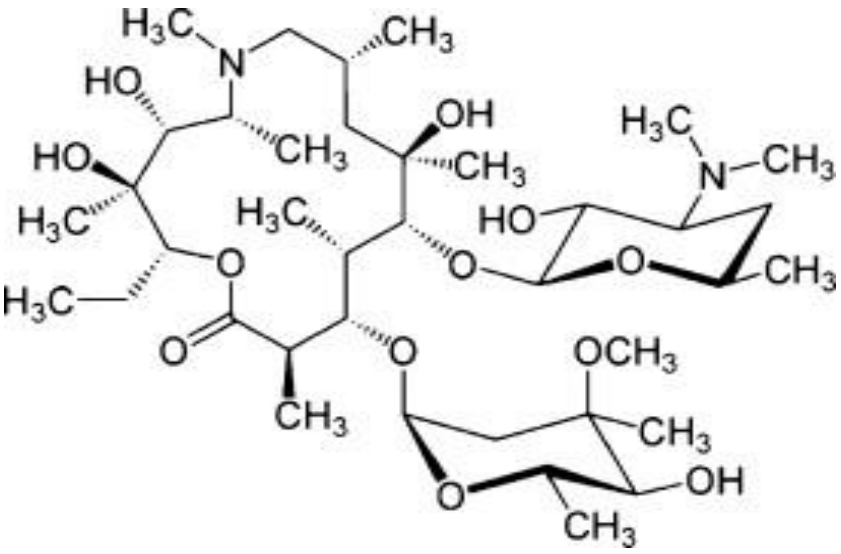

Figure 1 : Azithromycin chemical structure (Mohammadi et al., 2010).

Beyond its antibacterial activity, Azithromycin has shown antiviral and immunomodulatory activities that could be of interest in viral infections, including COVID-19 (Echeverría-Esnal et al., 2021). Also, has shown in vitro activity against a wide variety of viruses (Zika, Ebola, rhinovirus, enterovirus, influenza), with a wide range of 50% effective concentration, depending on cell culture and multiplicity of infection (Danesi et al., 2003; Horby et al., 2020; Tran et al., 2019)

Despite the development of new medicines, the treatment of intracellular infections frequently fails to eradicate the pathogens completely. An antibiotic should be delivered in a form that can be endocytosed by phagocytic cells and then released into these cells in order to effectively kill microorganisms. Antibiotics loaded into nanoparticles should deliver antibiotics to affected cells more effectively. Nanoparticles, which are colloidal in nature, biodegradable, and behave similarly to intracellular infections, were designed as carriers for these logistic targeting options (Mohammadi et al., 2010).

One major problem associated with azithromycin is its extremely poor solubility (belongs to BCS class II) in biological fluids which accounts for the poor bioavailability, upon its oral administration. Drugs that aren't well soluble in water may cause issues with drug efficacy. Most medications have low bioavailability because they are only soluble in a little amount of water. One of the most difficult challenges in medication development is

improving the dissolution and solubility of a poorly water-soluble drug. The nanoparticles formulation is one of the ways used to improve the solubility rate of poorly water-soluble drugs like azithromycin. To overcome the drug's poor bioavailability, azithromycin nanoparticles have been formulated into nanosuspensions (Zhang, Tan et al. 2007).

Nanoparticles as one of the most recent novel drug delivery carriers have been shown to improve drug efficiency via targeting the delivery of drugs, improving the bioavailability and either sustaining the release of the drugs or solubilizing them for systemic delivery. Additional advantage of this novel technology is the lower enzymatic as well as environmental degradation of pharmaceutical active ingredients (Shahira F. El-Menshawe et al., 2017; Gardouh et al., 2021; Ge et al., 2002).

The main features of nanoparticles, making them ideal candidates for drug delivery, are small size and use of biodegradable materials in their preparation. Indeed the nano-sized character of these particles causes their extravagation through the endothelium in inflammatory sites, epithelium, tumours, or penetrate microcapillaries and consequently allows for efficient uptake by a variety of cell types and selective drug accumulation at target sites (Gardouh et al., 2021; Singh & Lillard Jr, 2009; Sunderland et al., 2006).

Chitosan nanoparticles and their derivatives were chosen from among these nanoparticles since numerous researches have proposed that they are one of the best nanomaterials for providing antibacterial activity (Chandrasekaran et al., 2020). Chitosan is a naturally occurring cationic biopolymer made up of N-acetyl-D-glucosamine and D-glucosamine units linked together by β-1,4-glycosidic connections (Figure 2) (Goy et al., 2009; Kong et al., 2010). Previous research has looked into chitosan's antibacterial properties (Kong et al., 2010; Li et al., 2008; Rabea et al., 2003), and more recently, several forms of chitosan derivates have been made to improve its inherent antibacterial properties (Jia & Xu, 2001; Vinsova & Vavrikova, 2011; Wang et al., 2005; Yang et al., 2005).

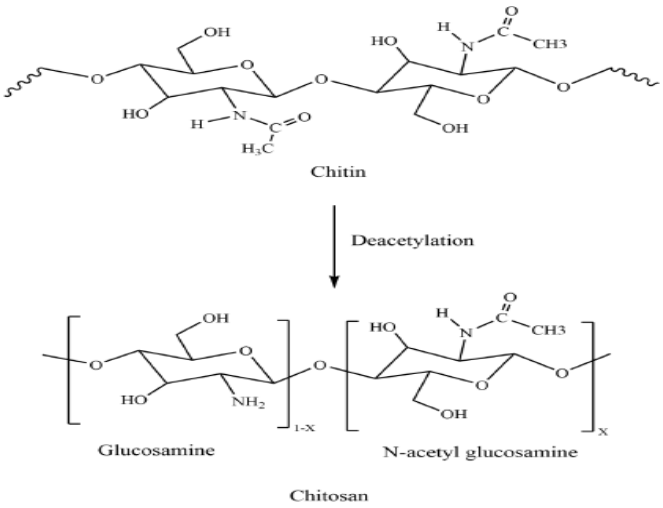

Figure 2: Schematic representation of chitosan (Chandrasekaran et al., 2020).

Biocompatibility (Hsu et al., 2011; Mi et al., 2002), biodegradability (Kim et al., 2011), nontoxicity (Shi et al., 2006), and antimicrobial activity (RabEa & StEuRbaut, 2010) are only a few of the outstanding biological properties of chitosan. These properties have made it helpful in a variety of industries, including medicine, food, agriculture, textiles, cosmetics, and others (Chandrasekaran et al., 2020; Kammoun et al., 2013).

Ionotropic gelation, microemulsion, emulsification solvent diffusion, polyelectrolyte complex, and the reverse micellar technique have all been used to produce chitosan nanoparticles (Divya & Jisha, 2018). Antibacterial activity has been demonstrated for nanoparticles made from diverse materials and composites. Antimicrobial (Ma et al., 2017; Perinelli et al., 2018), anticancer (Kravanja et al., 2019; Tokumitsu et al., 1999), anti-inflammatory, and antioxidant (MubarakAli et al., 2018; Zailani et al., 2021) properties of chitosan nanoparticles have been observed.

Due to the reported characters of chitosan nanoparticles, it was chosen to enhance azithromycin solubility and thus the bioavailability. So, the aim of the current research was to prepare azithromycin-loaded- chitosan nanoparticles by ionic gelation method to enhance azithromycin solubility and thus its antibacterial efficacy. The

prepared formulation will be characterized for particle size, polydispersity index, zeta-potential, in-vitro release, and in-vitro antibacterial activity against Escherichia coli microorganisms.

Materials and methods

Materials:

Azithromycin was supplied from Hikma pharmaceutical company, Egypt, Chitosan (100000- 300000 MW) was purchased form Sigma Aldrich, USA, Acetic acid was bought from fisher scientific, UK, Tripolyphosphate was purchased from Sigma Aldrich, USA, Dialysis membrane was obtained from SERVA electrophoresis GmbH, Germany (MWCO 12 kDa), Absolute ethanol, and sodium hydroxide were of analytical grade, Dibasic sodium phosphate $NaHPO_4$, Potassium dihydrogen orthophosphate KH_2PO_4, and triethanolamine were all the pharmaceutical grades and were used as received.

Methods:

1- Construction of Calibration Curve

All spectrophotometric measurements were carried out using a UV–Vis spectrophotometer (Acculab UVS, USA). The cells used for absorbance measurements were 1^{-}cm matched quartz cells. Samples were weighed by using an analytical balance (KERN – ABJ-NM/ABSN). The calibration curve was constructed based on validated methods reported (Malhotra et al., 2011) with a slight modification. Briefly, A stock standard solution was prepared by dissolving accurately weighted 20 mg of azithromycin in to a 10 ml absolute ethanol (used as a cosolvent as Azithromycin is not completely soluble in phosphate buffer pH 7.4) using volumetric flask to get concentration of 2 mg/ml. From the stock solution, samples were prepared to obtain serial concentration ranging from 10 µg/ml to 80 µg/ml using phosphate buffer pH 7.4. UV spectroscopy scanning (200-600 nm) was performed using the phosphate buffer pH 7.4 solution as blank to determine the λ_{max} of the azithromycin.

2- Preparation of Azithromycin-loaded-chitosan nanoparticles.

Azithromycin-loaded-chitosan nanoparticles was prepared according to the method described by Calvo et al (Fernández-Urrusuno et al., 1999) based on the ionic gelation of chitosan with tripolyphosphate anion. Briefly, 20 mg of azithromycin and 60 mg of Chitosan was dissolved in 30 ml of 1 % acetic acid aqueous solution. Under magnetic stirring at room temperature, 15 ml of 1.33 % sodium tripolyphosphate aqueous solution was added dropwise into the chitosan solution.

3- Particle size and polydispersity index determination

The chitosan nanoparticle loaded with azithromycin suspension were diluted with deionized water (0.01%, v/v) and the particle size and Polydispersity Index of the prepared nanoparticles were estimated using a Malvern size distribution analyzer (Zeta-sizer, Malvern Instruments, UK) at 25 °C. The refractive index and viscosity of water were 1.330 and 0.89 cp, respectively (Shahira F El-Menshawe et al., 2017).

4- Zeta potential

The chitosan nanoparticles loaded with azithromycin suspension were diluted with deionized water (0.01%, v/v) and the electrical charge of the prepared nanoparticles was measured using a Malvern size distribution analyser (Zeta-sizer, Malvern Instruments, UK) at 25 °C. The refractive index and viscosity of water were 1.330 and 0.89 cp, respectively (Gardouh et al., 2021).

5- Encapsulation efficiency

Encapsulation efficiency of the prepared formulation was measured by subjecting the formulation to centrifugation at 4 ˚C and 10000 rpm for 20 minutes using a refrigerated microcentrifuge (SIGMA 1-14 K, Osterode, Germany) (Shahira F El-Menshawe et al., 2017), and the unentrapped free azithromycin was diluted with phosphate buffer pH 7.4 and the Encapsulation efficiency was measured using the developed analytical method after filtering the sample through 0.22 mm filter. The trials were performed in triplicate and Encapsulation efficiency was calculated using the following equation.

$$\text{Encapsulation efficency \%} = \frac{\text{DT} - \text{DS}}{\text{DT}} \text{ X } 100$$

Where DT is the theoretical amount of Azithromycin, and DS is the detected or actual amount of azithromycin.

6- In-Vitro Drug-Release Study

In-vitro release of Azithromycin and azithromycin loaded chitosan nanoparticles was performed using the dialysis release method (Saddik et al., 2022) with some modification. Two mL of both azithromycin and Azithromycin -loaded chitosan nanoparticles suspension containing 889 µg of Azithromycin were placed on a dialysis membrane (MW cut off 12,000 Da) firmly stretched over one end of a glass cylindrical tube (3 cm^2 diameter). The tube was then immersed in a 100 mL beaker containing 50 mL phosphate buffer at pH 7.4 and placed in a thermostatic water bath at 37 °C and shaken at 100 rpm. At predetermined time intervals, 5 mL of sample were withdrawn and replaced by an equal volume of Phosphate buffer pH 7.4 to maintain the sink condition. The amount of Azithromycin released was determined by UV-spectrophotometer at wavelength 205.6 nm.

7- In-Vitro Antibacterial Activity

The in-vitro antibacterial activity of the azithromycin was initially evaluated using the agar diffusion method against clinical isolates of Escherichia coli. A freshly prepared bacterial suspension of 0.5 McFarland turbidity was inoculated on the surface of Mueller–Hinton agar plates and 1 cm wells were prepared using a cork borer. Agar was removed from the wells and azithromycin, as well as azithromycin loaded chitosan nanoparticles were added to the formed wells. Plates were incubated at 37 °C overnight and the diameters of the inhibition zones were observed (Allam et al., 2019)

Results and Discussion

1- Construction of Calibration Curve

Azithromycin is a macrolide antibiotic with a low solubility. Its low solubility is thought to be one of the main reasons for its low oral bioavailability (Aucamp et al., 2015). As a result of other researchers' solubility experiments, ethanol was chosen to dissolve azithromycin (Cao et al., 2020; Wang et al., 2014).

Standard calibration curve of azithromycin was constructed by dissolving the azithromycin in absolute ethanol firstly due to its poor water solubility then serial concentrations were prepared using phosphate buffer pH 7.4. The UV scan of standard solution of Azithromycin between 200-600 nm gives the absorption maximum at 205.6 nm (Figure 3).

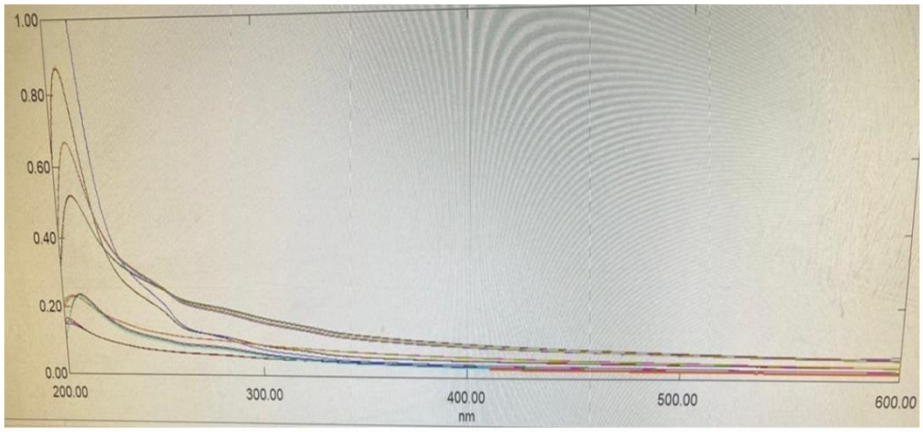

Figure 3: UV-Spectroscopic spectrum of Azithromycin in Phosphate Buffer pH 7.4.

The regression equation for azithromycin was obtained by plotting absorbance (A) versus concentration of azithromycin in the range of 10-80 µg/ml (Figure 4). The regression equation was y = 0.0105 x + 0.0439. The

regression coefficient (R^2 = 0.9968) was very much significant. The calibration curve obtained was evaluated by its correlation coefficient R^2 = 0.998. The LOD and LOQ of the proposed method were found to be 0.22 and 0.89 µg/ml, respectively.

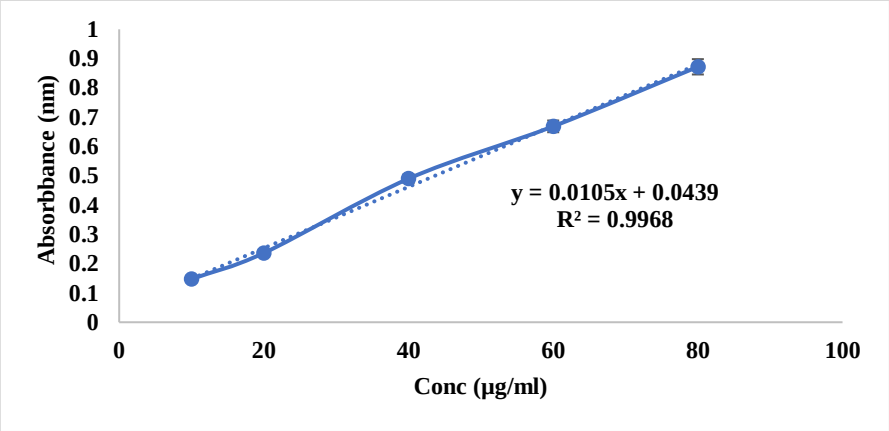

Figure 4: UV-Spectroscopic spectrum of Azithromycin in Phosphate Buffer pH 7.4 at wavelength of 205.6 nm.

2- Azithromycin-loaded-chitosan nanoparticles

Chitosan, a copolymer of N-acetylglucosamine and glucosamine derived from chitin, which has been included in European Pharmacopoeia and US Pharmacopeia. Owing to its biocompatibility, muco-adhesion and permeation-enhancing properties, chitosan has extensive applications in the medical field. More than a drug carrier for antibiotics delivery, chitosan has a wide spectrum of antimicrobial activity and high killing rate against Gram-positive and Gram-negative bacteria, but lower toxicity toward mammalian cells. They may be incorporated into fibers, membranes, or hydrogels, and used for contact disinfectants in many biomedical applications, including wound dressing, orthopaedic tissue engineering, drug-delivery carrier and haemodialysis (Kong et al., 2010)

Chitosan nanoparticles was chosen as chitosan is reported to have some advantages; that is, gel-forming ability at acidic medium, antacid and antiulcer activities that prevent or weaken drug irritation in stomach, the ability to swell and expand in the acid medium and enhancement toward the penetration of molecules across mucosal surface (Majithiya & Murthy, 2005). Chitosan microspheres for gastric mucoadhesive administration have been prepared and demonstrated to increase bioavailability of clarithromycin and tetracycline (Govender et al., 2005; Majithiya & Murthy, 2005)

Over the past few decades, the applications of nanotechnology have been widely used in many medical fields, especially in drug delivery. One of the areas that have received help from the advance is microbiology. Herein, we focus on the sustained-release role of nanoparticles for antibiotic delivery and materials used for the delivery. Owing to their ultrasmall size, nanoparticle formulations have many advantages over traditional dosage forms. As for the contribution to prolonged action of active agent, they can improve serum solubility of the drugs, prolonging the systemic circulation lifetime and releasing drugs in a sustained and controlled manner (Liu et al., 2009; Zhang et al., 2008). Moreover, antibiotic-loaded nanoparticles can enter host cells through endocytosis and then release drug payloads to treat microbe-induced intracellular infections (Zhang et al., 2010).

Ionic gelation (Calvo et al., 1997), reverse micellar technique (Brunel et al., 2008), microemulsion (Maitra et al., 1999), emulsion droplet coalescence (Tokumitsu et al., 1999), and spray drying (Davis & Illum, 1999) were all used to make CS-NPs. Physical crosslinking via ionic gelation was shown to be the most effective method among numerous. The electrostatic connection between a positively charged group of CS and a negatively charged group of tripolyphosphate is exploited in the ion gelation process (TPP). Furthermore, no chemical is utilized in the cross-linking process, which reduces hazardous side effects.

The azithromycin loaded chitosan nanoparticles was prepared according to method reported by Calvo et al. (Calvo et al., 1997). The method utilizes the electrostatic interaction between the positively amine group of chitosan and a negatively charged group of poly anion tripolyphosphate.

3- Particle size and polydispersity index determination

In comparison to its original predecessor, the smaller size of NPs has resulted in a considerable shift in physical properties. The particle size of azithromycin-loaded-chitosan nanoparticles (Figure 5) was found to be 276.5 nm with polydispersity index 0.357 indicating a successful nanoparticles preparation. The small particle size obtained increase surface area and increase penetration toward the cell wall of bacterial cells which leads to the efflux of intracellular molecules and bacterial cell death.

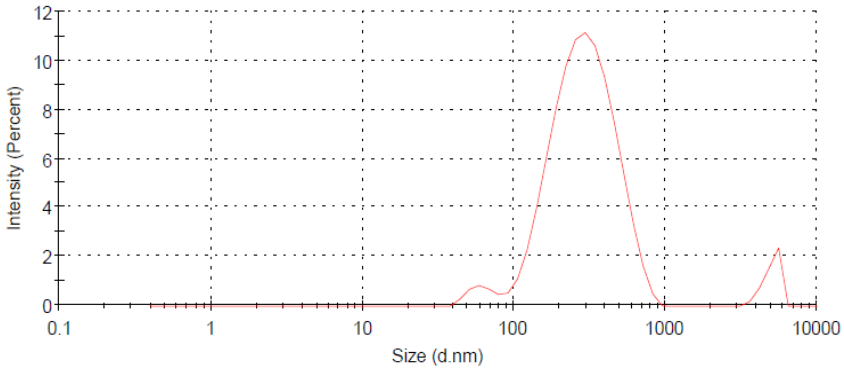

Figure 5: Particle size of Azithromycin loaded chitosan nanoparticles.

4- Zeta Potential

The Surface charge directly influences the aggregation behavior of the nanoparticles. The result of zeta potential of azithromycin- loaded- chitosan was + 25 mV suggesting good formulation stability (Honary & Zahir, 2013). The Positive charge obtained could be attributed to the fact that chitosan is the biopolymer that displays a cationic character due to the presence of its amino groups (NH_2) (Bernkop-Schnürch & Dünnhaupt, 2012; Younes & Rinaudo, 2015). The amino groups of glucosamine (positively charged) which can bind to the negatively charged bacterial cell surface and disrupt the normal functions of the membrane, e.g. by promoting the leakage of intracellular components or by inhibiting the transport of nutrients into cells (Du et al., 2009).

Nanoparticles having a positive charge have been found to be available technique for extending mucosa residence duration and ensuring continuous medication release. Positively charged particles can interact strongly with epithelia in numerous tissues, including the GI tract, because epithelial cells in various tissues, including the GI tract, have negative charges on their surface (Rojanasakul et al., 1992). So, it is expected that positively charged delivery systems can lead to extended residence time. The longer absorption time will ultimately result in better bioavailability of the azithromycin. The result of encapsulation efficiency was 80% indicating good encapsulation efficiency.

5- In-Vitro Drug-Release Study

The *in-vitro* drug release is an important parameter in the prediction of drug bioavailability. Rate of absorption and/or extent of bioavailability for such insoluble drugs are controlled by rate of dissolution in gastrointestinal fluids (Arora et al., 2010; Lipman, 1993).

It was observed that the cumulative amount released from azithromycin-loaded-chitosan nanoparticles was higher than obtained from azithromycin solution after three hours (Figure 6). Hence, the cumulative amount of dissolved drugs after three hours were 53.05 % for Azithromycin loaded chitosan nanoparticles and 7.25 % for azithromycin solution.

The results obtained could be attributed to the high particle surface area in contact with the medium might enhance the release of azithromycin which increase the solubility and release pattern.

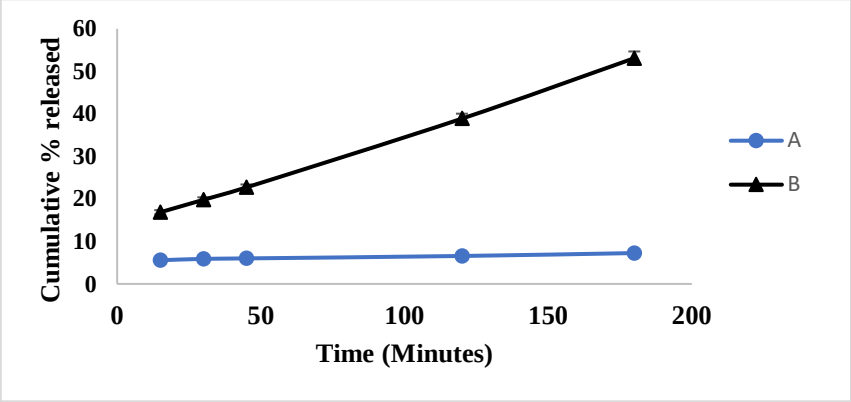

Figure 6: In-vitro release of (A) Azithromycin solution (B) Azithromycin-loaded-chitosan nanoparticles in phosphate buffer pH=7.4.

6- In- vitro antimicrobial activity

Recently, there has been a lot of interest in the antibacterial activity of chitosan nanoparticles and azithromycin. The agar diffusion method was used to determine the antibacterial activity of the formulations. The result of the in vitro antibacterial activity of the azithromycin against Escherichia coli was illustrated at (Figure 7). It was clearly observed that azithromycin loaded chitosan nanoparticles has a broader inhibition zone and higher antibacterial activity against Escherichia coli compared with azithromycin solution when used at concentration of 10 µg/ml. The reason behind this increase in inhibition zone could be explained by the antibacterial effect of chitosan nanoparticles and their small particle size which facilitate azithromycin penetration into bacterial cell wall causing bacterial cell death.

Another explanation may be due to the positive charge of azithromycin-loaded-chitosan nanoparticles which result in the electrostatic communication between the amino groups of glucosamine (positively charged) and the cell membrane of gram negative bacteria that contains lipopolysaccharide (LPS) which is comprised of anionic groups (phosphate and pyrophosphate groups) which provides more negative charges to the cell surface (Chandrasekaran et al., 2020; Tsai & Su, 1999). This contact causes widespread changes to the cell's surface, resulting in a change in membrane permeability, which causes osmotic imbalance and intracellular substance efflux, ultimately leading to cell death (Raafat et al., 2008; Shahidi et al., 1999). Furthermore, chitosan nanoparticles have been shown to enter bacterial cells, bind to DNA, and disrupt the replication machinery (Birsoy et al., 2015; Ivask et al., 2014). Flocculation of electronegative components in the cell by Chitosan causes the bacteria's physiological functions to be disrupted, resulting in bacterial cell death (El-Tahlawy et al., 2005).

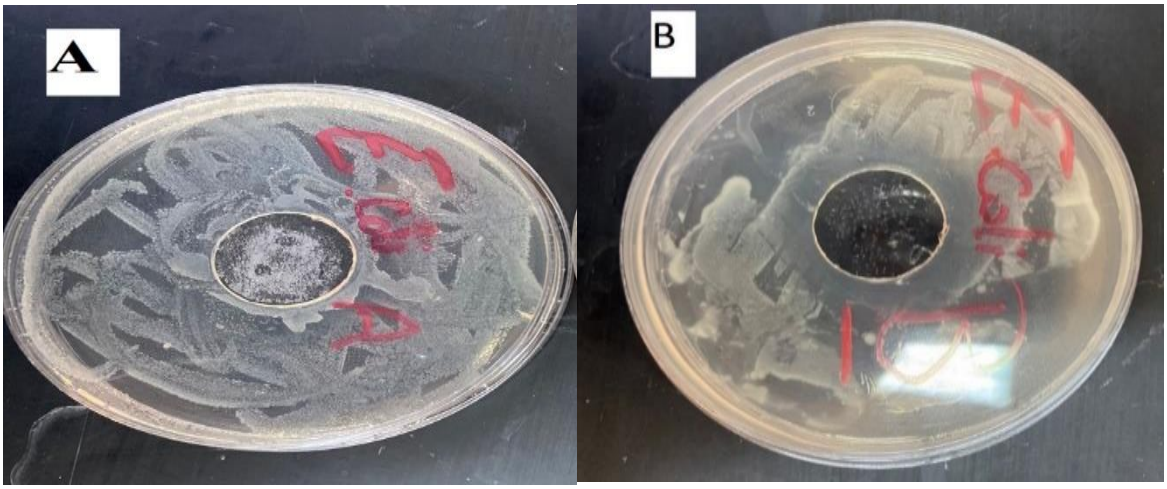

Figure 7: The mean inhibition zone diameters induced by A) Azithromycin solution B) Azithromycin-loaded chitosan nanoparticles in agar plates inoculated by Escherichia coli, and a representative figure of each treatment.

The figure shows that Azithromycin-loaded chitosan nanoparticles has superior anti-bacterial activities.

CONCLUSIONS

Chitosan, a natural biopolymer, has a wide range of antibacterial properties. It is possible to develop a highly active chitosan nanoparticles for a variety of industrial applications by applying appropriate nanoparticle synthesis processes. The biological use of chitosan nanoparticles, either alone or in combination with other chemicals, inhibits gramme negative bacteria such as E. coli. In this investigation the ionic gelation method was used for successful preparation of azithromycin- loaded-chitosan nanoparticles. The nanoparticles obtained were within nano-range with a positive charge which increase the contact time between azithromycin and bacterial cell wall. The amount of azithromycin released from the chitosan nanoparticles was more than 53 % in 3 hours compared with 7.25 % for azithromycin solution. The small particle size and large particulate surface area seems to be the main reasons for the increase in drug dissolution rate. The superior antibacterial activity of azithromycin-loaded- chitosan nanoparticles could be explained by several reasons including: the small particle size that facilitate azithromycin penetration, positive charge that increased contact time between nanoparticles and cell wall of bacteria, and the antibacterial effect of chitosan nanoparticles.

Finally, starch nanoparticles could be considered as a suitable technique for dissolution and antibacterial activity enhancement of azithromycin, which is a poorly soluble drug.

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