

**SYNTHESIS AND EVALUATION OF AMOXICILLIN AND
CEPHALEXIN ENCAPSULATED CHITOSAN NANOPARTICLES
AGAINST URINARY TRACT INFECTION CAUSING *ESCHERICHIA
COLI* AND *KLEBSIELLA PNEUMONIAE***

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ABSTRACT

Urinary tract infections (UTI) are persistent that leads to therapeutic failure because of poor penetration and limited availability of antibiotics. This study aims to detect the etiology of UTI and the antibiogram pattern to the commonly used antimicrobial agents in local scenario. The study also focused to develop an effective nanoformulation using chitosan against highly antibiotic resistant pathogens. Isolation, identification and evaluation of antibiogram by standard Kirby Bauer's method. The chitosan (CS) encapsulated resistant antibiotics was characterized by SEM, FTIR, drug loading, encapsulation efficiency, *in vitro* drug release and antibacterial effect against the resistant strains. 40 urine samples were processed, of which 60% were positive and was identified as *Escherichia coli* (47.5%), and

Klebsiella pneumoniae (12.5 %). The resistant rates of *E. coli* for amoxicillin (Amo) and cephalexin (Cep) were found to be 84.2% and 73.7% respectively. However, *K. pneumoniae* showed 100% resistance for both antibiotics. In accordance, we developed a biocompatible antibiotics encapsulated with chitosan for their sustained release into the bacterial cells. This was confirmed by disc diffusion method which showed susceptible zones with Amoxicillin-chitosan nanoparticles (Amo-CS Nps) and cephalexin-chitosan nanoparticles (Cep-CS Nps) when compared to bare Amoxicillin and cephalexin. Thus the development of such a nanoformulation could be used for the better treatment of wide range of other bacterial diseases and in developing safe vehicles for drug delivery in the future.

Keywords : Amoxicillin, Chitosan, Cephalexin, Nanoparticles, UTI.

INTRODUCTION

UTI represents one of the prevalent microbial diseases, frequent in the outpatient as well as in the nosocomial setting and their financial burden on society is significant. UTI are the common cause of morbidity in women [1]. Majority of UTI are not life threatening and do not cause any irreversible damage. Gram negative bacteria such as enterobacteriaceae species including *Escherichia coli*, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Enterobacter agglomerans*, *Citrobacter freundii* account for over 70% of UTI cases [2,3] and the most common pathogen is *E. coli* [4]. In complicated UTI and hospitalized patients, *Enterococcus faecalis* and *Pseudomonas* spp. are comparatively more common. The relative frequency of the pathogens varies depending upon age, sex, catheterization, and hospitalization [5].

Higher incidence of UTI in the general population, complications involved in high risk groups and the cost involved in treatment, emphasizes the importance of appropriate antibiotic therapy. However, microbial resistance to nearly all classes of antimicrobials continues to rise in spite of increasing awareness and concerns worldwide [6-8]. Resistant rates among gram-negative bacteria varied from 56.5-82.6% against trimethoprim/sulfamethoxazole, tetracycline, Amoxicillin & ampicillin. Among gram positive isolates, resistance rates ranged from 50-100% against tetracycline, trimethoprim-sulphamethoxazole, Amoxicillin and penicillin-G [9]. The main cause of this issue is the improper and uncontrolled use of antibiotics [10] and inappropriate dosage [11].

Thus resistant to many currently used antibiotics demand for long-term solutions to this ever-growing and predictable problem. The efficacy of many drugs is often limited by their potential to reach the site of therapeutic action due to various problems such as poor bioavailability, *in vivo* stability, solubility, intestinal absorption, sustained and targeted delivery to site of action, therapeutic effectiveness; side effects, and fluctuations of drug concentration in plasma which either fall below the minimum effective concentrations or exceed the safe therapeutic concentrations [12]. One of the recent breakthroughs in addressing antimicrobial resistance lies in exploring antimicrobial nanoparticles and novel nanosized platforms, for efficient antibiotic delivery. Compared to conventional antibiotics, antimicrobial Nps offer many distinctive advantages in reducing acute toxicity, overcoming resistance and lowering cost of treatment [13, 14]. Such a drug delivery system can be achieved by encapsulating the active ingredient in a polymer matrix such that drug could find

its way through the bloodstream for longer duration with reduced dose and frequency of administration [15, 16]. Earlier reports showed that entrapment of antibiotics such as ciprofloxacin, ofloxacin and Amoxicillin trihydrate in polymeric nanoparticles could effectively target intracellular pathogens and increased the efficacy of the antibiotic several fold against infections [17-20].

Recently chitosan, a natural polymer (poly β -(1-4)-2-amino-2-deoxy-D-glucose), is being used as an effective drug delivery devices owing to its non-toxicity, biodegradability, biocompatibility, mucoadhesion and antibacterial and low cost [21]. Studies have confirmed that chitosan and chitosan oligosaccharide-grafted membranes showed antibacterial activity against *Escherichia coli*, *Pseudomonas aeruginosa*, methicillin-resistant *Staphylococcus aureus* [22]. Scientist have also observed that the antimicrobial activity of chitosan and graft copolymers against *Candida albicans*, *Trichophyton rubrum*, and *Trichophyton violaceum* depends largely on the amount and type of grafted chains, as well as on the changes in pH [23]. The goal of the present study is to enumerate the etiology of UTI, study their antibiogram pattern and to develop a nanoparticle based targeted delivery system for antibiotics against *E.coli* , and *K. pneumoniae* by ionic gelation method. The developed systems were characterized for their shape, chemical interaction, drug loading, entrapment efficiency, *in vitro* drug release and antibacterial activity of Amo/Cep-CS Nps against Amo and Cep resistant UTI pathogens.

1. MATERIALS AND METHODS

1.1. Materials

Media, chemicals and antibiotics (ampicillin, Amoxicillin, cephalexin, ceftazidime, amikacin, gentamicin, ciprofloxacin, norfloxacin, cotrimoxazole, and nitrofurantoin) used in this study were purchased from Hi-Media Laboratories, India.

1.2. Isolation and identification of bacteria from UTI

Patients were asked to collect mid-stream urine samples using sterile, wide mouthed container. Patient's name, age, and time of urine collection were indicated on the container. Female patients between 18 and 65 years of age with community-acquired UTI were included in the study. Urine specimens were processed in the laboratory within 2 hours of collection and specimens that were not processed within 2 hours were kept refrigerated at 4 °C until it was processed.

Loopful of a well mixed urine sample was streaked on MacConkey and blood agar plates and incubated at 37°C aerobically for 24h. The plates were then examined macroscopically for bacterial growth. The bacterial colonies were counted and multiplied by 100 to give an estimate of the number of bacteria present per milliliter of urine. A significant bacterial count was taken for specimens that produced $\geq 10^5$ colonies.

Identification of isolates was done according to the standard bacteriological techniques. Colony morphology, hemolytic pattern, Gram reaction and microscopic features were used as primarily identification criteria. Biochemical tests, namely indole, citrate, oxidase, catalase, H₂S production, lysine decarboxylase, reaction on triple sugar iron agar, lactose fermentation, urea hydrolysis and gas production were performed for identification of Gram negative bacteria. Catalase test, coagulase test and haemolysis pattern on blood agar were used for identification of Gram positive bacteria.

1.3. Antibigram by disc diffusion method

Antimicrobial susceptibilities of the bacterial isolates were performed according to the criteria of Clinical Laboratory Standards Institute (CLSI, 2005) using the Kirby–Bauer disc diffusion method on Muller-Hinton Agar. 2-3 colonies from a pure culture plate was touched with wire loop and inoculated into peptone water medium. It was incubated at 37°C for 2 hours, and turbidity of the suspension was adjusted to the turbidity of McFarland 0.5 standard and swabbed on Muller Hinton agar medium (MHA). The following antibiotic discs were tested: ampicillin (10µg), Amoxicillin, cephalixin, ceftazidime, amikacin (30µg), gentamicin (10µg), ciprofloxacin (5µg), norfloxacin (10µg), co-trimoxazole (25µg), and nitrofurantoin (300µg). MHA plates were incubated at 37°C for about 18 to 24 hours and the zones of inhibition were measured and results were interpreted based on CLSI standards (CLSI, 2005). The standard reference strain *E. coli* (ATCC 25922) was used to assure testing performance of the potency of antibiotic discs. Ethical clearance was obtained from Sathyabama University ethical review committee. Only participants who gave their consent were included in the study.

1.4. Preparation of antibiotics loaded chitosan nanoparticles

Chitosan nanoparticles (CS Nps) were prepared by ionic gelation method. 0.1g of chitosan was dissolved in 1 % v/v acetic acid solution. 12ml of 0.4% Sodium tripolyphosphate (TPP) solution was added drop wise with a syringe to CS solution while stirring, followed by sonication for 20 min. The resulting suspension was subsequently centrifuged at 10,000 rpm

for 20 min. The pellets obtained were resuspended in deionised water by sonication. Amo (30µg) and Cep(30µg) was dissolved in sterile distilled water. Drug-loaded chitosan nanoparticles were formed spontaneously upon drop wise addition of drug solution to chitosan-TPP suspension under magnetic stirring, followed by sonication. The resulting nanosuspensions were centrifuged 4 times (20 min each) at 10,000 rpm, washed with distilled water and dried.

1.5. SEM and FTIR

The surface morphology was determined by scanning electron microscopy (SEM JEOLJSM-6490LA). The chemical interaction of Amo and Cep within CS Nps was studied using Perkin-Elmer Spectrum RX1 Fourier transform infrared spectrophotometer.

1.6. Determination of drug loading and entrapment efficiency

Amoxycilin and cephalexin was standardized in ethanol at 270 nm using UV-vis spectrophotometer and standard graph was obtained. Amo-CS and Cep-CS nanosuspension was centrifuged at 20,000 rpm and supernatant was collected. The encapsulation efficiency of nanoparticles was determined by extracting the unentrapped Amo and Cep remaining in the supernatant with ethanol and quantified spectrophotometrically. The drug loading and encapsulation efficiency was calculated as:

$$\text{Drug loading (\%)} = \frac{\text{Total amount of Amo and Cep} - \text{Free Amo and Cep} \times 100}{\text{weight of nanoparticles measured after freeze drying}}$$

$$\text{Encapsulation efficiency (\%)} = \frac{\text{Total amount of Amo and Cep} - \text{Free Amo and Cep} \times 100}{\text{Total amount of Amo and Cep}}$$

1.7. *In vitro* drug release study

In vitro release of Amo and Cep from CS Nps was studied in PBS (pH 7.4) using pellet dispersion method. Amo and Cep in CS Nps pellet was suspended in PBS, pH 7.4 at a final concentration of 0.5 mg/ml. The nanosuspension was equally distributed in different eppendorf tubes and aliquots of samples were collected at desired time intervals from 0h to 28h and centrifuged at 20,000 rpm for 1h to separate the nanoparticles. Amo and Cep released in the supernatant was measured spectrophotometrically using ethanol as baseline at 270 nm. Based on the standard equation obtained for Amo and Cep from the standard graph, concentrations of Amo and Cep released at different time points was calculated. All the experiments were done in triplicate.

1.8. Assessment of antibacterial activity of Amo-CS/Cep-CS Nps

The strains which showed resistant to Amo and Cep were used in this study. Disc diffusion technique was used. 6mm sterile filter paper was dipped with Amo-CS and Cep-CS nanosuspension with the control of bare Amo and Cep and placed on to the agar plate which was streaked with the *E. coli* and *K. pneumoniae*. Plates were incubated for 24 h at $37 \pm 2^\circ\text{C}$ and zone of inhibition was determined to evaluate the bacterial inhibition.

1.9. Minimum inhibitory concentration (MIC) of bare Amoxicillin/cephalexin and Amo CS/Cep-CS Nps

MIC of bare Amoxicillin/cephalexin and Amo/Cep-CS Nps were determined in Muller Hinton (MH) broth medium using standard protocols [24].

2. RESULTS AND DISCUSSION

2.1. Isolation of bacteria from UTI

Nowadays, drug resistance to urinary isolates is a severe concern and seeks for an urgent medical attention in community [24]. At the same time, the associated global financial crisis requires the validation of antimicrobial prescriptions in order to reduce morbidity and mortality. The most effective management of UTI is to identify the pathogens and selecting effective antimicrobial agent against them [25]. In this study, of the 40 urine samples processed from patients with UTI 60% (24/40) were positive for culture showing significant bacteriurea [26, 27]. All the isolates were gram negative bacilli, which were identified as *E.coli* (19/40, 47.5%) and *K. pneumoniae* (5/40, 12.5%) (Fig. 1) and is in agreement with other reports [28-30].

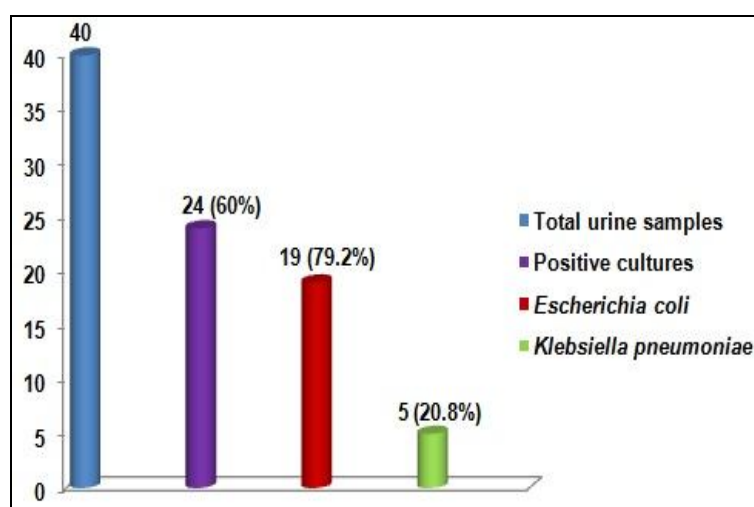


Fig. 1 Percentage of bacterial isolates from urine samples

2.2. Antibigram by Kirby Bauer method

Results of antimicrobial susceptibility tests showed marked differences in their susceptibility pattern. According to Fig. 2a nitrofurantoin was found to be most susceptible drug in 96.7% cases of *E. coli* followed by amikacin (84.5%), norfloxacin (73.7%), gentamicin (68.4%) and co-trimoxazole (52.6%). However 84.2% of strains were resistant to Amoxicillin followed by 73.7% to cephalixin, 57.9% to ampicillin, 52.6% to ceftazidime and 47.4 % to ciprofloxacin. *K. pneumoniae* showed 100% resistance to amoxycilin and cephalixin followed by 60% resistance to ampicillin, ceftazidime and gentamicin. On the other hand, all the isolates were 100% susceptible to amikacin and ciprofloxacin and 80% were susceptible to nitrofurantoin and co-trimoxazole followed by 70% susceptibility to norfloxacin (Fig. 2b). Thus, from the observed results it is clear that nitrofurantoin showed excellent activity against all *E. coli* isolated in the present study and can be recommended in empirical treatment of community-acquired UTI [31]. Recently, Pignanelli *et al.* demonstrated highest susceptibility (>90%) to imipenem, nitrofurantoin, cephalosporins and aminoglycosides in community-acquired UTI [32]. The antimicrobial resistance patterns are valuable guide to practical treatment [33]. Akram *et al.*, from Aligarh reported highest resistance to ampicillin and co-trimoxazole among enteric gram negative bacilli [34]. Higher resistance rates to ampicillin (>80%) and Amoxicillin (>95%) was reported by Beyene *et al.* in Ethiopia for *E. coli* and *K. pneumoniae* [9]. Resistance rates of more than 85% was reported by Han *et al.* for *E. coli* and *K. pneumoniae* to ampicillin and most cephalosporins [35]. A similar pattern was also observed in Nepal where *E. coli* showed 88.3% and 57.1% resistance to ampicillin, and cephalixin and higher resistance to ampicillin (100%) and cephalixin (80%) for *Klebsiella spp* was also reported by Baral *et al.* [36].

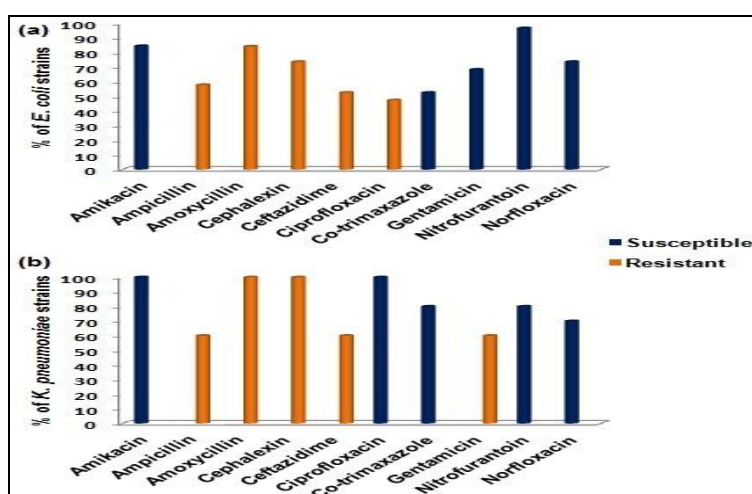


Fig. 2 Antibigram of tested antibiotics against (a) *E.coli* and (b) *Klebsiella pneumoniae* strains

2.3. Morphology of Amo-CS and Cep-CS nanoparticles

SEM analysis of Amo-CS and Cep-CS nanoparticles (Fig. 3a & b) showed them as small spherical clumps and with clear cross linking.

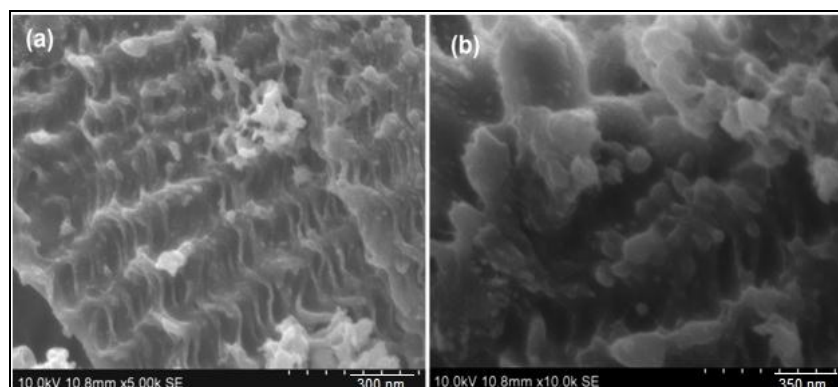


Fig. 3 SEM photographs (a) Amo-CS Nps (b) Cep-CS Nps

2.4. Fourier Transform Infra Red Spectroscopy

Fig. 4 shows the FTIR spectrum of the prepared nanoparticles revealing the chemical interaction between the components. The FT-IR spectrum of CS-Nps (Fig. 4a) showed stretching vibrations of amine and hydroxyl groups at 3420 cm^{-1} . The peaks found at 1740 and 1629 cm^{-1} correspond to the stretching vibrations of carbonyl and protonated amino groups with a peak due to C-O stretching at 1320 cm^{-1} . However, in Amo-CS and Cep-CS nanoparticles (Fig. 4b & c), no absorption peak that corresponds to carbonyl groups was found (1740 cm^{-1}). On the other hand the presence of strong band at 1639 cm^{-1} and less intense peak at 1320 cm^{-1} was confirmed which can be attributed to the stretching vibrations of TPP cross-linked carboxyl groups of CS. Thus a shift in the peak at 1639 cm^{-1} in CS nanoparticles to 1609 cm^{-1} in Amo-CS and Cep-CS nanoparticles with a variation in the transmittance intensity was established for the presence of Amo-CS and Cep-CS nanoparticles [37, 38].

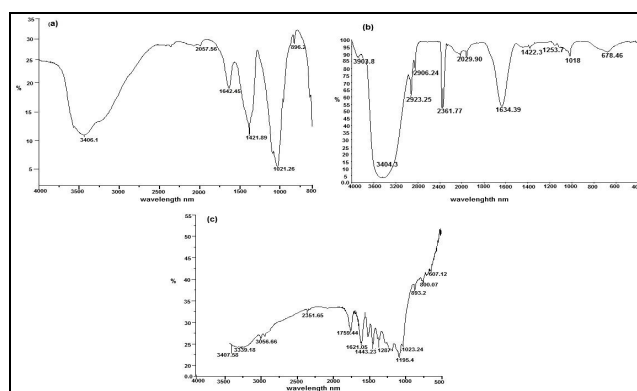


Fig. 4 FTIR pattern for (a) Chitosan nanoparticles (b) Amo-CS Nps and (c) Cep-CS Nps

2.5. Drug loading and entrapment efficiency

The effort to enhance the loading of Amo and Cep in CS nanoparticles had significant effect in the present work. As shown in Table 1 the highest drug content was 24%. The addition of Amo and Cep extensively improved the drug loading efficiency of the nanoparticles due to the stronger interaction between antibiotics and CS. The encapsulation efficiency of the prepared nanoparticles was 78%. In the present work, the influence of chitosan concentration and Amo and Cep entrapment in nanoparticles was evaluated. The highest encapsulation efficiency was found in the formulations prepared with the lowest amount of chitosan. Chitosan concentration affected considerably the encapsulation and drug loading efficiencies in particular the loading capacity increased as the chitosan concentration decreased. This is because the increase in chitosan concentration led to increased solution viscosity that decreased the loading capacity of the nanoparticles as a consequence of the reduced drug [39, 40].

Table 1 Loading and encapsulation paramaters of Amo-CS and Cep-CS Nanoparticles

Chitosan nanoparticles (CS Nps) (mg)	Antibiotics (mg)		Drug Loading (%)	Encapsulation Efficiency (%)
	Amoxicillin (Amo)	Cephalexin (Cep)		
15	2	2	7.6	43.1
10	2	2	17	57.6
5	2	2	24	78

2.6. *In vitro* drug release from chitosan

In vitro drug release of Amo and Cep from the Amo-CS and Cep-CS Nps (Fig. 5) showed that 58.2% of Amo and 61% Cep was released within 24h followed by almost sustained release of 65% after 48h. The initial release may be attributed to the drugs that are bounded to the surface and a sustained release after 18h which may be due to solubility nature of chitosan leading to swelling and degradation. Thus such a nanoformulation of antibiotics encapsulated with polymers may perhaps recover the bioavailability of the drug and could conquer the restrictions of unstablity and Amo/Cep resistance, toxicity and loss due to quick clearance and metabolism.

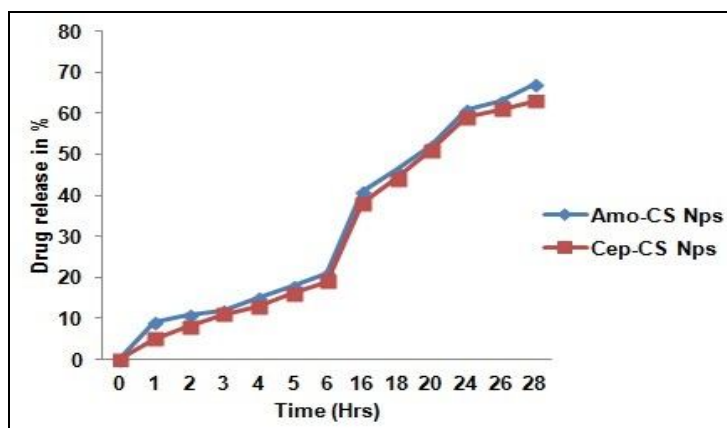


Fig. 5 – *In vitro* release of Amoxicillin and cephalixin from chitosan nanoparticles

2.7. Antibacterial effect of Amoxicillin and cephalixin encapsulated with and without chitosan against the resistant strains of *E.coli* and *Klebsiella pneumoniae*.

According to the literature [41] chitosan possess antimicrobial activity against Gram-negative and Gram-positive bacteria. *E.coli* (84.2%) and *K. pneumoniae* (100%) strains which were resistant to bare Amoxicillin were tested with Amoxicillin coated with chitosan. All the strains which were resistant to bare Amoxicillin showed susceptible zones with Amoxicillin-chitosan combination. Similarly, *E.coli* (73.7 %) and *K. pneumoniae* (100 %) strains which showed resistance to cephalixin were also tested on chitosan coated cephalixin. All of them showed susceptible zone sizes (Fig. 6).

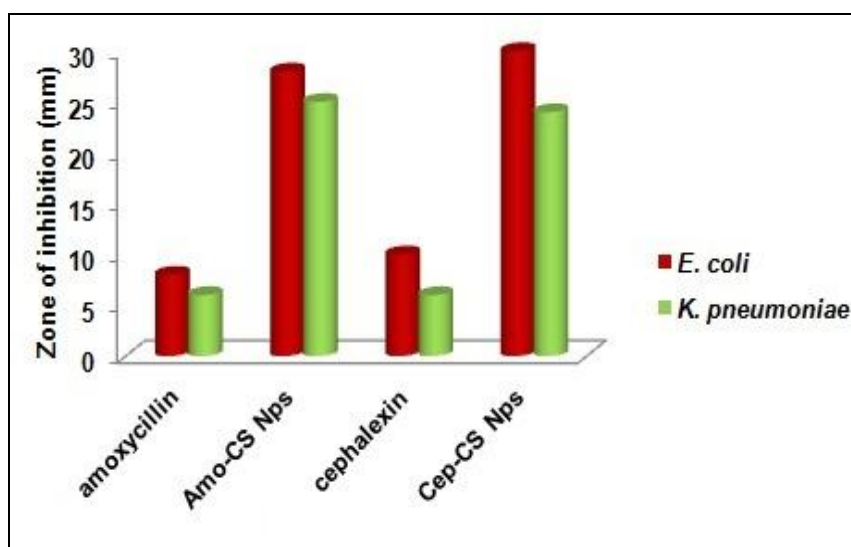


Fig. 6 Zone of inhibition for *E. coli* and *K. pneumoniae* against chitosan encapsulated amoxicillin and cephalixin compared with amoxicillin and cephalixin discs

2.8. Minimum Inhibitory Concentration for Amoxicillin and Cephalexin encapsulated with and without Chitosan

The MIC values of bare Amo and Cep for *E.coli* and *K. pneumoniae* was found to be 64 µg/ml whereas MIC values for antibiotic encapsulated chitosan was 2 µg/mL (Amo-CS) and 4 µg/mL (Cep-CS) for *E.coli*. Similarly, MIC values for antibiotic encapsulated chitosan was 4 µg/mL (Amo-CS) and 8 µg/mL (Cep-CS) for *K. pneumoniae* (Fig. 7). It was well known that encapsulated antibiotics could effectively increase the maximal tolerated dose and target the intracellular pathogens when compared with the free antibiotics [42-44]. Partha and his co workers have reported that the Ampicillin trihydrate-loaded chitosan nanoparticles resulted in a superior antimicrobial activity compared to bare nanoparticles, probably due to the synergistic effect of chitosan and ampicillin trihydrate [45]. Furthermore, non-pegylated liposome increased the efficacy of the vancomycin in sufficiently higher concentrations (120-fold) inside the intracellular compartments of macrophages and showed increased bactericidal activity against methicillin resistant *S. aureus* [46].

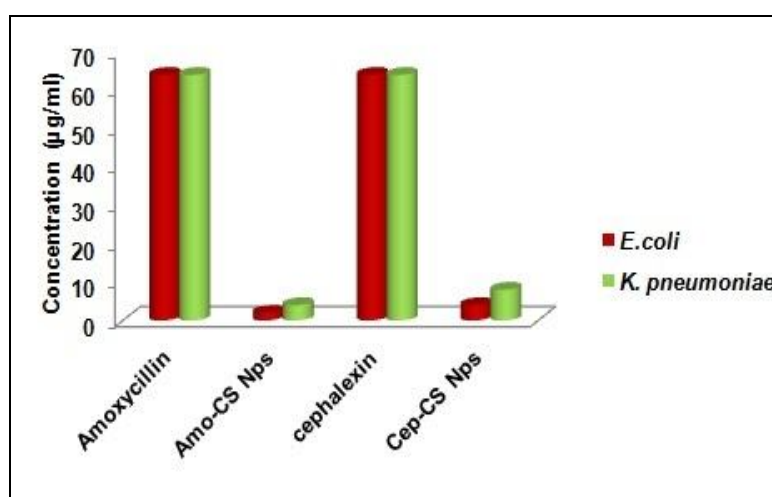


Fig. 7 – MIC for Amoxicillin and Cephalexin antibiotics encapsulated with and without chitosan nanoparticles

CONCLUSION

The present study confirms *E. coli* and *K. pneumoniae* as the predominant pathogen in urinary tract infections. The antibiotics (Amo/Cep) found to be highly resistant towards the UTI pathogens showed an effective antimicrobial activity by encapsulating them with chitosan developed by ionic gelation method (Amo/Cep-CS Nps). In accordance, MIC value of Amo/Cep-CS Nps showed susceptible MIC values whereas bare Amo/Cep resulted in resistant values. Furthermore, the encapsulated Amo/Cep-CS Nps also exhibited sustained

drug release behavior and thus improved the bioavailability of the drug. Finally, the antibiotics loaded nanoparticles has promising antibacterial properties which may be a new strategy to overcome the breadth of multidrug resistant isolates of UTI.

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