Research Paper

Development and Characterization of Green Tea Catechins and Ciprofloxacin-loaded Nanoemulsion for Intravaginal Delivery to Treat Urinary Tract Infection

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The aim of the present study was to develop a nanoemulsion-based delivery system containing green tea catechins called polyphenon 60 and ciprofloxacin for intravaginal delivery to treat urinary tract infection. Polyphenon 60 and ciprofloxacin were encapsulated in a single nanoemulsion system prepared using ultrasonication technique. The nanoemulsion was characterized by determining particle size, zeta potential, morphological structure and estimating *in vitro* release and antibacterial efficacy. To determine the *in vivo* pharmacokinetic parameters and intravaginal transportation of nanoemulsion in Sprague Dawley rats, gamma scintigraphy and biodistribution study was conducted with technetium pertechnetate-labelled nanoemulsion. The preliminary antibacterial investigation showed synergy between these compounds with FIC_{index} of 0.42. The developed formulation showed zeta potential of +55.3 mV and globule size of 151.7 nm, with polydispersity index of 0.196. The percent *in vitro* release for polyphenon 60 at the end of 7 h was 94.8±0.9, whereas for ciprofloxacin it was 75.1±0.15 in simulated vaginal media. Antibacterial activity evaluation against extended spectrum beta lactamase and metallo beta lactamase strains revealed that nanoemulsions containing 4 and 10 mg/ml each of polyphenon 60 and ciprofloxacin effectively inhibited the growth of bacterial strains. In biodistribution study, the percent radiolabelled drug per gram was found to be 3.50±0.26 and 3.81±0.30 in kidney and urinary bladder, respectively at 3 h. From these findings it could be concluded that the developed polyphenon 60+ciprofloxacin nanoemulsion showed antibacterial activity against *Escherichia coli* and was transported efficiently to the target organs through vaginal mucosa.

Key words: Ciprofloxacin, gamma scintigraphy, intravaginal drug delivery, nanoemulsion, polyphenon 60

Ciprofloxacin (CF) is a broad spectrum antibiotic of fluoroquinolone class. It has been widely used for treating urinary tract infection (UTI) caused by *Escherichia coli*, however, development of resistance has been reported[1]. In a recent study, CF resistance was reported to be 49.9 % against *E. coli*[2]. Jaktaji and Pasand[3] in a recent paper have reported that CF efficiency has decreased due to development of fluoroquinolone resistant strains. The problem of bacterial resistance to antimicrobial drugs has been attempted to be resolved by combining antibiotics with natural plant products. Jazani and Babazadeh[4] reported possible synergy between CF and aqueous green tea extracts when used for antibacterial action. Polyphenon 60 (P60) is a natural compound present in green tea (*Camellia sinensis*). Polyphenols exhibit strong antioxidative and free radical scavenging activities[5]. P60 irreversibly damages the cytoplasmic membrane of bacteria and acts as a potential antiadhesive agent. However, it has been reported that Gram-negative bacteria are less susceptible to catechins as lipopolysaccharides act as a barrier[6]. In a previous study, it was also reported that catechins inhibited the activity of dihydrofolate reductase, an enzyme

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required by pathogens for the synthesis of purines and pyrimidines and increased the epidermal thickness[7]. Roccaro *et al*.[8] have also reported synergistic antibacterial action of green tea with various antibiotics including tetracycline, β-lactams and fluoroquinolone. Lee *et al*.[9] evaluated the synergistic effect of catechins with CF on the treatment of chronic bacterial prostatitis in a rat model. Catechins combined with CF showed synergistic action and a significant inhibition of bacterial growth in chronic bacterial prostatitis-induced rats.

In the present study, CF was combined with green tea catechins and formulated as a nanoemulsion for testing against extended spectrum β-lactamase (ESBL) and metallo-β-lactamase (MBL) producing bacterial strains. The strains selected for the study were antibiotic-resistant clinical isolates comprising of *E. coli*, *Klibsiella pneumonia*, *Proteus mirabilis* and *Citrobacter* sp.

The oral bioavailability of P60 and CF is 26 % and 69 %, respectively, which is reported to be low because of high first pass effect[10,11]. To provide for a spatial and temporal control, vaginal route of administration was explored. Prolonged residence time, biocompatibility, biodegradability were some of the key features for an ideal vaginal formulation. Oil in water (O/W) nanoemulsions are easy to prepare and scale up, have tendency to solubilize both hydrophilic and lipophilic compounds owing to oil and water phases and have their own antimicrobial effect. All these features in addition to small particle size make nanoemulsion an attractive carrier system to solubilize and transport actives across various mucosal barriers. Besides, nanoemulsions are known to provide stability to the encapsulated actives, this feature is in particular importance when encapsulating natural compounds as these are prone to oxidation and degrade/destabilize as soon as they come in contact with biological fluids. It was hypothesized that a nanoemulsion-based formulation for intravaginal (IVAG) administration would provide stability to the encapsulated CF and green tea and the nanometric size of the droplets would facilitate transport of the active across the vaginal mucosa into systemic circulation. Tuğcu-Demiröz *et al*.[12] compared vaginal hydroxypropylmethylcellulose-based bioadhesive gels of oxybutynin with commercial oral tablets and found higher area under curve (AUC) and bioavailability with vaginal administration of bioadhesive gels.

To study the transport of formulation across vaginal mucosa; P60 was radiolabelled with technetium pertechnetate (^{99m}Tc) and its path was studied in a comparative oral vs. IVAG gamma scintigraphy and biodistribution study. The ^{99m}Tc-P60+CF nanoemulsion was administered (IVAG and oral) to female Sprague Dawley rats and scintigrams were taken. Pharmacokinetic parameters were investigated in the blood and target organs like kidney, urinary bladder and spleen in Sprague Dawley rats after oral and IVAG administration.

MATERIALS AND METHODS

CF hydrochloride was generously gifted by Torge Ltd. Labrasol (caprylcaproyl macrogol glyceride*)* was kindly gifted by Gatefosse (India). Trolox was purchased from Calbiochem (unit of Merck Millipore, India). Nutrient dehydrated agar and nutrient dehydrated broth were obtained from Qualigens, India. P60, fetal bovine serum and Dulbecco's modified Eagle medium were purchased from Sigma-Aldrich. All the other chemicals used in the study were of analytical grade or HPLC grade.

E. coli (MTCC 739) for preliminary studies was obtained from the Microbial Type Culture Collection and Gene Bank, Chandigarh, India. The bacterial culture was maintained in nutrient broth. These isolates were maintained on Luria-Bertani agar slants and stored under refrigerated conditions.

Approval to carry out animal studies was obtained from the Institute of Nuclear Medicine and Allied Sciences (INMAS), Institutional Animal Ethics Committee (IAEC), New Delhi, India, IAEC vide number INM/IAEC/2012/05 and their guidelines were followed throughout the study. Female Sprague Dawley rats (aged 3-4 mo) weighing 180-200 g were obtained from the Central Animal House Facility of INMAS, Delhi, India. Rats were kept at normal room temperature of 25±5°.

Preliminary screening of antibacterial potential of P60 and CF:

Stock solutions of aqueous P60 (6.6 mg/ml), CF (20 µg/ml) and P60+CF (6.6+20 µg/ml) were prepared and diluted in the nutrient broth. To account for the effect of P60 and CF on bacterial viability, nitroblue tetrazolium (NBT) assay was performed. The wells containing 100 µl of each dilution and *E. coli* bacterial inoculum (5×10^5 cfu/ml) was incubated for 16 h at 37°. After incubation for 16 h, 25 µl of NBT (5 mg/ml) was added to the wells and plates were further incubated for 3-4 h. About 100 µl dimethyl sulfoxide

was added to each well to stop the reaction and dissolve the blue crystals. To account for the colour effect of P60 and CF, absorbance (Abs) was measured at 595 nm using enzyme-linked immunosorbent assay reader at the beginning of the assay and after completion of incubation[13]. The mean percent inhibition was calculated according to the Eqn. 1[13]: % inhibition = (1–((Abs of sample at t_{16} −Abs of sample at t_0)/(Abs of growth control at t_{16}–Abs of growth control at t_0))×100). The value which exhibited more than 90 % inhibition was used as the minimum inhibitory concentration (MIC). Fractionated inhibitory concentration (FIC) for P60+CF in aqueous form was calculated using a checkerboard method. FIC for P60+CF in aqueous form was calculated using Eqn. 2: $FIC = MIC_{A+B}/MIC_A$; $FIC_B = MIC_{B+A}/MIC_B$; $FICI = FIC_A+FIC_B$. Here, MIC_{A+B} was the MIC of drug A in combination with B and MIC_{B+A} was vice versa. If the FIC_{index} was ≤0.5, the combination was defined as synergistic and additive if FIC_{index} value was between 0.5-1[14].

Preparation of P60+CF nanoemulsion:

To select specific excipients for preparing final emulsion, solubility of P60 and CF was checked in different oils, surfactants and co-surfactants. P60+CF nanoemulsion was prepared by mixing 20 mg/ml P60 and 20 mg/ml CF in 10 % v/v labrasol as oil, 80 % v/v of 1 % cetyperidinium chloride in water as surfactant, 10 % v/v glycerol as co-surfactant to form a pre-emulsion. This pre-emulsion was subjected to homogenization at 10 000 rpm for 25 min using high speed homogenizer (Tissue Master 125 homogenizer, Omni International, Georgia) and subsequently to high energy ultra-sonication via Bench Top Ultrasonicator (Model UP400S, 24 KHz 400 W, Hielcher, Ultrasound Technology, Germany) for the time period of 150 s, at an amplitude of 40 % and pulse of 30 % (i.e. 0.3 s ON and 0.7 s OFF) to prepare the final P60+CF nanoemusion.

Determination of globule size:

Globule size distribution was determined by using photon correlation spectroscopy with inbuilt Zetasizer (model: 100 HS, Malvern Instruments. UK). Before measuring size and zeta potential, the developed nanoemulsion was diluted to 1:50 v/v in HPLC water[15].

Transmission electron microscopy (TEM) of nanoemulsion:

Morphological structure of developed P60+CF nanoemulsion was determined by using TEM. A drop of diluted (1:50 v/v) sample of nanoemulsion was placed on a carbon-coated copper grid (Quantifoil, Germany) and left to dry. A drop of negative stain uranyl acetate (2 % w/v) was used to stain the sample. The grid was again air dried and examined under TEM. The sample was viewed under Jeol JEM-1230 TEM operating at 80 kV.

***In vitro* release studies:**

About 2 ml of developed P60+CF nanoemulsion was packed in the dialysis bag (cellulose membrane mw. cut-off 12400, Sigma) immersed in 500 ml simulated vaginal medium (acetate buffer with pH 3: was prepared by dissolving sodium acetate 13.6 g and 6 ml until pH 3 of acetic acid in 1000 ml of distilled water)[12] and subjected to stirring at 100 rpm at 37° in USP II dissolution apparatus. The samples were withdrawn from the vessel and the drug release was determined spectrophotometrically for CF at 316 nm and for P60 at 274 nm.

Antibacterial studies against bio-safety level-2 strains:

The effect of nanoemulsions on the test pathogens (*E. coli*, *K. pneumoniae*, *P. mirabilis*, *C. amalonatus*, *C. diversus*) was assayed by agar well diffusion method. The agar diffusion method was used to determine the minimal bactericidal concentration (MBC) of nanoemulsions. The bacterial culture was diluted to a final concentration of 1×10^6 cfu/ml and was plated on pre-prepared Muller-Hinton agar. Different concentrations of nanoemulsion (2, 4 and 10 mg/ml) were made and 10 µl was added to each well (0.5 mm) bored on agar plates. Further, the plate was incubated at 37° for 24 h and zones of inhibition were measured[16]. MBC was taken as the lowest concentration of nanoemulsion that completely inhibited the growth of pathogens[16].

Radiolabeling of P60+CF nanoemulsion with ^{99m}Tc:

Radiolabeling of P60 with ^{99m}Tc pertechnetate was done using direct labelling method[17,18]. About 500 µl of P60-Sol (3 mg P60 in acetone) was taken and mixed with 200 µl of $SnCl_2$ dihydrate solution (2 mg/ml in ethanol). To the resultant mixture (filtered through 0.22 µ nylon 66 membrane filter), required volume of sterile ^{99m}Tc-pertechnetate (5 mCi) was added with continuous mixing such that it had a radioactivity of 5 mCi/ml. The resultant formulations obtained had 100 µCi/20 µl activities. The radiolabelled P60 was then

mixed with the CF nanoemulsion and was incubated at 30±5° for 30 min. The radiochemical purity of ^{99m}Tc-P60+CF nanoemulsion was determined using ascending instant thin layer chromatography (Gelman Sciences, Inc., Ann Arbor, MI, USA) using acetone as mobile phase. The effect of concentration of $SnCl_2$ and incubation time on radiolabeling efficiency was studied to achieve optimum reaction conditions by using the Eqn. 4. *In vitro* stability of radiolabeled formulation was evaluated and optimized in normal saline as well as in blood plasma[19,20]. Stable radiolabeled formulation was then subjected to gamma scintigraphy and biodistribution studies. Eqn. 4: % radiolabelling = radioactive counts retained in lower half of strip/total radioactive counts retained in the strip×100.

The Sprague Dawley female rats were selected for the gamma scintigraphy study. The rats were divided in three groups (3 rats for each formulation, n= 9). Group-I: rats administered P60+CF nanoemulsion (IVAG); group-II: rats administered P60+CF nanoemulsion (orally); group-III: rats administered aqueous P60+CF (IVAG).

Rats were anesthetized using 0.4 ml ketamine hydrochloride intraperitonial injection (50 mg/ml). Radiolabeled formulation ^{99m}Tc-P60+CF nanoemulsion and aqueous form (0.6 µl) with the concentration of (100 µCi/20 µl) equivalent to (20 mg/kg and 7 mg/kg body weight of P60 and CF, respectively) was administered orally and IVAG with the help of catheter made up of low density polyethylene tubing of internal diameter 0.1 mm at the delivery site respectively to the rats. Anesthetized rats were then placed on the imaging platform and imaging was performed at pre-determined time point of 0.5, 3, 6 and 24 h using a single-photon emission computerized tomography gamma camera, SPECT, LC 75-005, Diacam, Siemens AG, Erlanger, Germany.

Further biodistribution study was conducted on female rats. Similar groups were selected as in gamma scintigraphy studies and 3 rats were selected per time point in each group (n= 36). Before the administration of formulations, the rats were anesthetized using 0.4 ml ketamine hydrochloride intraperitoneal injection (50 mg/ml). Blood samples were collected through retro-orbital vein puncture and rats were sacrificed by cervical dislocation at predetermined time points (0.5, 3, 6 and 24 h) post-administration of formulations. Subsequently, urinary tract organs (kidney, spleen and urinary bladder along with ureters) were dissected, washed twice using normal saline, made free from adhering tissue/fluid, and weighed. Radioactivity present in each tissue/organ was measured using shielded well-type gamma scintillation counter. Radio pharmaceutical uptake per gram in each tissue/organ was calculated as a fraction of administered dose using Eqn. 5: radioactivity (%/g of tissue) = counts in sample×100/weight of sample×total counts injected.

Pharmacokinetic parameters for P60+CF nanoemulsion were calculated[21]. Organ targeting efficiency for kidney and urinary bladder was calculated using two Eqns. 6 and 7 mentioned below. Drug targeting efficiency (DTE %) that represents time average partitioning ratio was calculated using Eqn. 6: DTE (%) = [(AUC target organ/AUC blood))$_{IVAG}$/(AUC target organ/AUC blood)$_{oral}$]×100.

Direct transport percent (DTP %) of target organ was calculated using Eqn. 7: DTP (%) = [B_{IVAG}– Bx/B_{IVAG}]×100, where Bx = (B_{oral}/P_{oral})×P_{IVAG}, Bx is the target organ AUC fraction contributed by systemic circulation following oral administration, B_{oral} is the $AUC_{0–24h}$ (target organ) following oral administration, P_{oral} is the $AUC_{0–24h}$ (blood) following oral administration, B_{IVAG} is the AUC_{0-24h} (target organ) following IVAG administration, P_{IVAG} is the AUC_{0-24h} (blood) following IVAG administration, AUC is the area under the curve.

Data analysis:

Results of *in vitro* drug release and biodistribution data were reported as mean±SD (n= 3), and the difference between the groups were tested using two-way ANOVA using GraphPad Prism 5.0 and data analysis tool in Microsoft Excel.

RESULTS AND DISCUSSION

The antimicrobial activity of P60 and CF was quantified by calculating the MIC, the lowest concentration at which the agent inhibits the growth of the pathogen for *E. coli*[14]. The results (Table 1) indicated that CF is highly effective against *E. coli* at lower concentrations (20 ng/ml) as compared to P60 (3 mg/ml). Hoshino *et al*.[22] also demonstrated that catechin-copper (II) complexes did damage to the cytoplasmic membrane of *E. coli*, which was an important mechanism in killing *E. coli*. Ikigai *et al*.[23] demonstrated that catechins induced leakage of molecules from the intra-liposomal space by disrupting the membrane, suggesting that this action is responsible for bactericidal activity of catechin against *E. coli*. However, on combining CF

and P60, the MIC was reduced up to three folds as compared to individual MIC. FIC_{index} value against *E. coli* was found to be 0.424 and the combination can said to be synergistic (FIC_{index}<0.5). The results were in agreement with Lee *et al*.[9] where it was reported that combination of catechins and CF was more effective than CF alone in treating chronic bacterial prostatitis. Isogai *et al*.[24] evaluated the synergistic effect of catechins and levofloxacin in mouse intestine with *E. coli* infection. It was suggested that combination of P60 with CF was effective for eliminating the bacteria and able to reduce the rate of reoccurrence of infection.

In the present work, the nanoemulsion was prepared and loaded with 20 mg/ml of CF and 20 mg/ml of P60. The loaded nanoemulsion was characterized using particle size analysis and zeta potential. The results (Table 2) showed average particle size of both placebo and drug-loaded nanoemulsion was 142.7 and 151.7 nm, respectively. This indicated that the nanoemulsions approached a monodisperse stable system. Based on previous study reported by Neves *et al*.,[25] this size range of 100 to 200 nm was selected as potentially optimal in terms of vaginal drug delivery, particularly when trying to target the epithelial layer. The zeta potential of placebo and drug-loaded nanoemulsion was +39.7 and +55.3 mV, respectively indicating a high stability of droplets against coalescence of dispersed phase.

The negative stained TEM picture of this o/w nanoemulsion was shown in fig. 1. The image represented the particle size (around 150 nm), which appeared in agreement with the measured particle size by zetasizer. In addition, these electron microscopy pictures showed good monodispersity of the particles, thus indicating the relatively good quality of formulation.

Dissolution studies done in simulated vaginal medium at pH 3 showed percent release of P60 to be 94.8± 0.9 % and of CF to be 75.1±0.15 % at 7 h (fig. 2). Owing to high aqueous solubility, the release of both P60 and CF was high[26]. The release was found to be sustained for a period of 7 h. Both the compounds showed prolonged release in vaginal media, which proves that both P60 and CF were soluble at vaginal acidic pH and the nanoemulsion could be able to control the release in a sustained manner[25].

Gram-negative resistant isolates were collected from different health care centers of Mumbai and were identified using conventional cultural, biochemical and morphological tests, and comprised of *E. coli*, *K. pneumoniae*, *Proteus mirabilis*, *C. amalonatus*, *C. diversus*[16]. MBC was identified and the findings demonstrated that in both ESBL and MBL-producing isolates, the P60+CF nanoemulsion showed no inhibition of growth of all the isolates at 1:10 dilution containing 2 mg/ml of each P60 and CF whereas growth was inhibited at 1:5 and 1:2 dilutions containing 4 and 10 mg/ml of each P60 and CF, respectively. The placebo showed no growth inhibition of all strains at 1:10 and 1:5 dilutions whereas effective inhibition of growth was observed at 1:2 dilution. Therefore, the 1:5 and

TABLE 1: PRELIMINARY SCREENING OF THE POLYPHENON 60+CIPROFLOXACIN NANOEMULSION

Preliminary screening of P60+CF			
MIC (P60)	3.3 mg	MIC (ciprofloxacin)	20 ng
MIC (P60 in combination)	0.412 mg	MIC (ciprofloxacin in combination)	6 ng
FIC (aq.P60)	0.412/3.30= 0.124	FIC (aqueous ciprofloxacin)	6/20= 0.3

FIC_{index}= $FIC_{aq.\ P60}$+$FIC_{aq.\ ciprofloxacin}$= 0.124+0.3= 0.424. MIC is minimum inhibitory concentration, FIC is fractional inhibitory concentration, P60 is polyphenon 60

TABLE 2: CHARACTERIZATION OF THE NANOEMULSION FORMULATION

	Drug concentration (mg/ml)	MDS (nm)	PDI	Zeta potential (mV)
Placebo	-	142.7	0.433	+39.7
Nanoemulsion (ciprofloxacin+P60)	20+20	151.7	0.196	+55.3

MDS is mean droplet size, PDI is polydispersity index

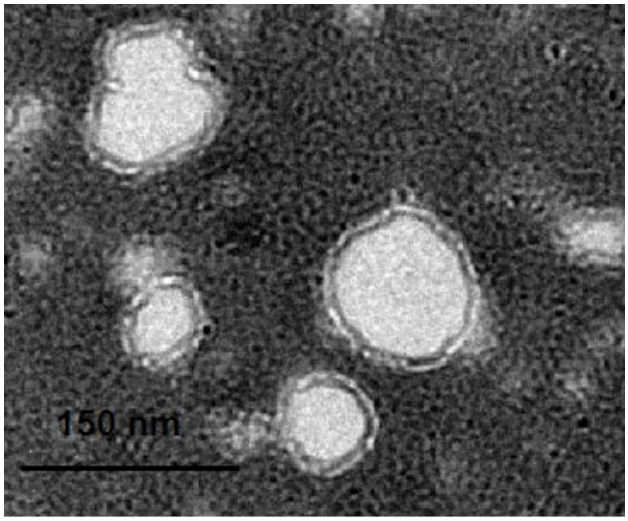

Fig. 1: TEM with a negative staining technique
TEM coupled with a negative staining technique of a diluted sample of polyphenon 60 and ciprofloxacin nanoemulsion. Staining agent: uranyl acetate

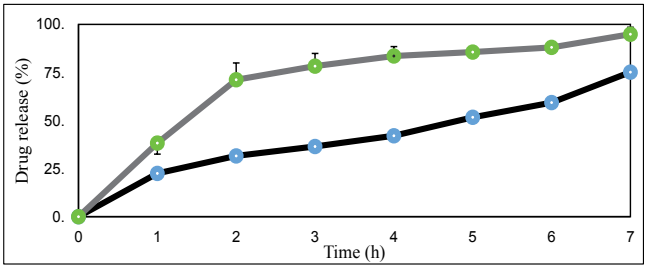

Fig. 2: *In vitro* drug release of ciprofloxacin and P60 form the optimized nanoemulsion

***In vitro* drug release of ciprofloxacin (CF, —●—) and P60 (—●—) form the optimized nanoemulsion in simulated vaginal media**

1:2 dilutions containing 4 and 10 mg/ml of each drug were effective for the growth inhibition (Table 3). The results indicated that both P60 and CF are potent against ESBL and MBL-producing uropathogens. Higher antimicrobial effect of nanoemulsions can be attributed to the formation of nanodrops that increase the surface tension and thereby force themselves to merge with the lipids present in the bacterial cell membrane[27]. On a mass scale, this effectively disintegrates the membrane and kills the bacteria. Moreover, water present in nanoemulsion system is tightly bound to the internal oil phase and therefore not available to bacteria for its growth[28]. Aruna *et al*.[16] also observed in the study that uropathogens showed higher degree of resistant towards antibiotics. It also revealed that (72.05 %) ESBL produces isolates were resistant to CF, one of the most commonly used fluoroquinolone drugs for UTI. But our results demonstrated that CF when combined with natural green tea catechins in a single nano carrier system showed inhibition of resistant strains.

P60+CF nanoemulsion was used to label with ^{99m}Tc because of its suitable chemical properties for labelling process. P60+CF nanoemulsion was successfully labelled with ^{99m}Tc and labelling efficiency of ^{99m}Tc-P60+CF nanoemulsion and ^{99m}Tc-P60+CF aqueous form was 99.7±1.3 and 94.4±0.9 %, respectively. The distribution and retention time of radiolabelled P60+CF nanoemulsion was studied by gamma scintigraphic images of Sprague Dawley rats after IVAG and oral administration at predetermined time points of 0.5, 3, 6 and 24 h. Scintigrams of urinary tract of rat administered with P60+CF nanoemulsion by IVAG route showed the presence of drug in kidney, urinary bladder and vaginal tract for 6 h and trace amounts at 24 h and maximum distribution at 3 h (fig. 3). Whereas images of rat administered with P60+CF nanoemulsion orally showed distribution of drug in gastric tract and images of female rats, which received IVAG P60+CF aqueous form showed less distribution of drug in urinary tract (fig. 3). The literature[29] suggested that it was due to the permeation of nano droplets across the vaginal mucosa. Our results were in agreement to previous study of Neves *et al*.,[25] where fluorescent images of the deprivin-loaded nanoparticles administered (IVAG) in female mice were taken and higher uptake of drug was detected at vaginal level and in uterine tissue. Woolfson in 2003[30] also observed in a study that radiolabelled microspheres given intravaginally in sheep model showed higher retention time up to 12 h in the urinary tract.

Further, biodistribution studies of ^{99m}Tc-P60 CF nanoemulsion and ^{99m}Tc-P60+CF aqueous form following IVAG and oral administration in Sprague-Dawley rats were performed, and the radioactivity in percent per gram of organ of administered dose was estimated at predetermined time intervals up to 24 h (Table 4). The concentration of radiolabelled drug in blood at all sampling time points for different formulations was also assessed and recorded (fig. 4A). The analysis indicated that the percent per gram concentration of ^{99m}Tc-P60+CF nanoemulsion in kidney and urinary bladder following the IVAG administration (figs. 4B and C) were found to be 3.50±0.26 and 3.81±0.30 at 3 h, which was significantly higher as compared to both ^{99m}Tc-P60+CF nanoemulsion administered orally (1.19±0.12 and 0.88±0.16) and ^{99m}Tc-P60+CF aqueous form administered intravaginally (1.21±0.11 and 1.34±0.12). While the spleen concentration (fig. 4D) of ^{99m}Tc-P60+CF nanoemulsion after IVAG administration was comparable to that of oral administration of ^{99m}Tc-P60+ CF nanoemulsion and IVAG administration of ^{99m}Tc-P60+CF aqueous form at all the time points. Tedajo *et al*.[31] also demonstrated the applicability of emulsions as a new drug carrier system for vaginal delivery with a good retention of the emulsion in the vagina. The pharmacokinetic parameters for the ^{99m}Tc-P60+CF nanoemulsion and ^{99m}Tc-P60+CF aqueous form were calculated (Table 5). The lower T_{max} values (3 h) for target organs after IVAG administration when compared to oral administration (6 h) may also be attributed to preferential target drug delivery following IVAG administration of ^{99m}Tc-P60+CF nanoemulsion. This confirmed that ^{99m}Tc-P60+CF nanoemulsion crossed epithelium rapidly and reached the target organs when given intravaginally as compared to oral administration. When the C_{max} and AUC of kidney and urinary bladder concentration of ^{99m}Tc-P60+CF

TABLE 3: ANTIBACTERIAL POTENTIAL OF P60+CIPROFLOXACIN NANOEMULSION AGAINST BsI-2 STRAINS (ESBL AND MBL PRODUCING UROPATHOGENS)

Antibacterial activity of nanoemulsions on pathogens							
Activity of various dilutions of nanoemulsions against ESBL cultures							
		P60+ciprofloxacin nanoemulsion (dilutions)			Placebo (dilutions)		
		1:10	1:5	1:2	1:10	1:5	1:2
250	*E. coli*	+	-	-	+	+	-
253	*E. coli*	+	-	-	+	+	-
254	*E. coli*	+	-	-	+	+	-
255	*E. coli*	+	-	-	+	+	-
258	*E. coli*	+	-	-	+	+	-
260	*E. coli*	+	-	-	+	+	-
261	*E. coli*	+	-	-	+	+	-
262	*E. coli*	+	-	-	+	+	-
263	*E. coli*	+	-	-	+	+	-
264	*E. coli*	+	-	-	+	+	-
210	*K. pneumoniae*	+	-	-	+	+	-
275	*K. pneumoniae*	+	-	-	+	+	-
296	*K. pneumoniae*	+	-	-	+	+	-
466	*K. pneumoniae*	+	-	-	+	+	-
507	*K. pneumoniae*	+	-	-	+	+	-
610	*K. pneumoniae*	+	-	-	+	+	-
645	*K. pneumoniae*	+	-	-	+	+	-
674	*K. pneumonia*	+	-	-	+	+	-
9b	*P. mirabilis*	+	-	-	+	+	-
110	*P. mirabilis*	+	-	-	+	+	-
150	*P. mirabilis*	+	-	-	+	+	-
191	*P. mirabilis*	+	-	-	+	+	-
15	*C. diversus*	+	-	-	+	+	-
30	*C. diversus*	+	-	-	+	+	-
59	*C. amalonatus*	+	-	-	+	+	-
123	*C .diversus*	+	-	-	+	+	-
Activity of various dilutions of nanoemulsions against MBL cultures							
95	*K. pneumoniae*	+	-	-	+	+	-
101	*K. pneumoniae*	+	-	-	+	+	-
457	*K. pneumoniae*	+	-	-	+	+	-
202	*K. pneumoniae*	+	-	-	+	+	-
462	*K. pneumoniae*	+	-	-	+	+	-
614	*K. pneumoniae*	+	-	-	+	+	-
618	*K. pneumoniae*	+	-	-	+	+	-
85	*P. aeruginosa*	+	-	-	+	+	-
214	*P. aeruginosa*	+	-	-	+	+	-
216	*P. aeruginosa*	+	-	-	+	+	-
463	*E. coli*	+	-	-	+	+	-
467	*E. coli*	+	-	-	+	+	-
469	*E. coli*	+	-	-	+	+	-
471	*E. coli*	+	-	-	+	+	-
473	*E. coli*	+	-	-	+	+	-
479	*E. coli*	+	-	-	+	+	-
493	*E. coli*	+	-	-	+	+	-
519	*E. coli*	+	-	-	+	+	-
520	*E. coli*	+	-	-	+	+	-
522	*E. coli*	+	-	-	+	+	-
525	*E. coli*	+	-	-	+	+	-

526	*E. coli*	+	-	-	+	+	-
621	*E. coli*	+	-	-	+	+	-
623	*E. coli*	+	-	-	+	+	-

ESBL is extended spectrum ß-lactamases, MBL is metallo-ß-lactamases. (dilution 1:10 contains 2 mg/ml each P60 and ciprofloxacin, dilution 1:5 contains 4 mg/ml each P60 and ciprofloxacin and dilution 1:2 contains 10 mg/ml each P60 ciprofloxacin). '+' Sign indicates no inhibition of bacterial growth whereas '-' sign indicates effective inhibition of bacterial growth

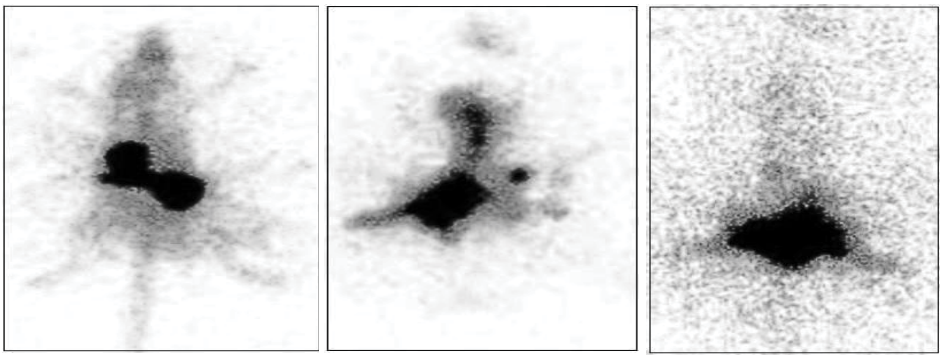

Fig. 3: Gamma scintigraphy images
(A) Oral ^{99m}Tc-P60+ciprofloxacin nanoemulsion, (B) IVAG ^{99m}Tc-P60+ciprofloxacin NBG, (C) IVAG ^{99m}Tc-P60+ciprofloxacin aqueous form showing presence of radioactivity in different organs

TABLE 4: DISTRIBUTION OF ^{99M}TC-P60+CIPROFLOXACIN NANOEMULSION (ORALLY AND IVAG), ^{99M}TC-P60+CIPROFLOXACIN AQUEOUS FORM (IVAG) AT DIFFERENT TIME INTERVALS IN SPRAGUE DAWLEY FEMALE RATS

Formulation and route of administration	Distribution of radiolabelled P60+ciprofloxacin in counts/g of tissues in different organs at different sampling time points				
	Organ	0.5 h	3 h	6 h	24 h
Oral ^{99m}Tc-P60+ciprofloxacin nanoemulsion	Blood	0.75±0.25	1.08±0.12	1.23±0.22	0.42±0.11
	Kidney	0.82±0.20	1.19±0.12	1.89±0.14	0.80±0.13
	Urinary bladder	0.38±0.08	0.88±0.16	1.34±0.11	0.65±0.10
	Spleen	0.59±0.15	0.97±0.23	1.62±0.10	0.33±0.09
IVAG ^{99m}Tc-P60+ciprofloxacin nanoemulsion	Blood	0.52±0.10	1.22±0.21	1.9±0.16	1.08±0.22
	Kidney	1.65±0.15*	3.50±0.26*	2.92±0.24*	1.30±0.18*
	Urinary bladder	2.92±0.32*	3.81±0.30*	3.22±0.25*	1.91±0.14*
	Spleen	0.41±0.18	0.90±0.30	1.05±0.29	0.50±0.15
IVAG ^{99m}Tc P60+ciprofloxacin aqueous form	Blood	0.4±0.31	0.74±0.10	0.92±0.08	0.33±0.09
	Kidney	0.82±0.09	1.21±0.11	1.50±0.12	0.54±0.09
	Urinary bladder	2.10±0.20	1.34±0.12	1.04±0.09	0.62±0.13
	Spleen	0.26±0.07	0.52±0.20	0.59±0.21	0.20±0.09

Each value is the mean±SD of three estimations. Radioactivity was measured at 0 h and all the measurements were performed using 0 h sample corresponding the organ as blank sample. Only statistically significant outcomes at P<0.05 have been reported. Asterisk mark showed significant values

nanoemulsion (IVAG), ^{99m}Tc-P60+CF nanoemulsion (orally) and ^{99m}Tc-P60+CF aqueous form (IVAG) were compared, the C_{max} kidney (3.50 %/g) and C_{max} urinary bladder (3.81 %/g) and AUC kidney (54.28 h %/g) and AUC urinary bladder (65.85 h %/g) of ^{99m}Tc-P60+CF nanoemulsion (IVAG) were found to be significantly higher. This could be due to small size of particles and large surface area of nanoemulsion. Vaginal absorption and bioavailability of multiple emulsions were studied and it was observed that the application of multiple emulsions may facilitate application, and reduce irritation and burning in the vulvovaginal region as compared to tablets formulations[32-34]. The % DTP and DTE represent the percent of drug directly transported to the target organs via the IVAG pathway. DTP and DTE % were calculated using tissue/organ distribution data following IVAG and oral administration and are recorded in Table 6. ^{99m}Tc-P60+CF nanoemulsion (IVAG) showed the highest DTE % kidney (106.29), DTE % urinary bladder (175.40) and DTP % kidney (54.5), DTP % urinary bladder (43.416) for kidney and urinary bladder amongst ^{99m}Tc-P60+CF nanoemulsion (oral) and ^{99m}Tc-P60+CF aqueous form (IVAG). The higher DTP % and DTE % suggest that ^{99m}Tc-P60+CF

nanoemulsion (IVAG) has better target organ efficiency and the observations were in sync with the reports of Cu *et al*.[35] who observed that nanoemulsion particles were found at significantly higher concentration in the reproductive tissues, beyond the mucus layer, at 2 and 6 h after IVAG delivery. Vaginal drug delivery bypasses the first pass effect; avoiding the gastrointestinal degradation and hepatic metabolism. Cicinelli *et al*.[36] also reported that radiotracer was absorbed through vaginal mucosa and raised its concentration in the paravaginal spaces, lymph, and vaginal venous vessels and reached ultimately in the systemic circulation. Similarly, it was observed that ^{99m}Tc-P60+CF nanoemulsion travelled efficiently throughout the target organs and reached systemic circulation and remained active up to 24 h.

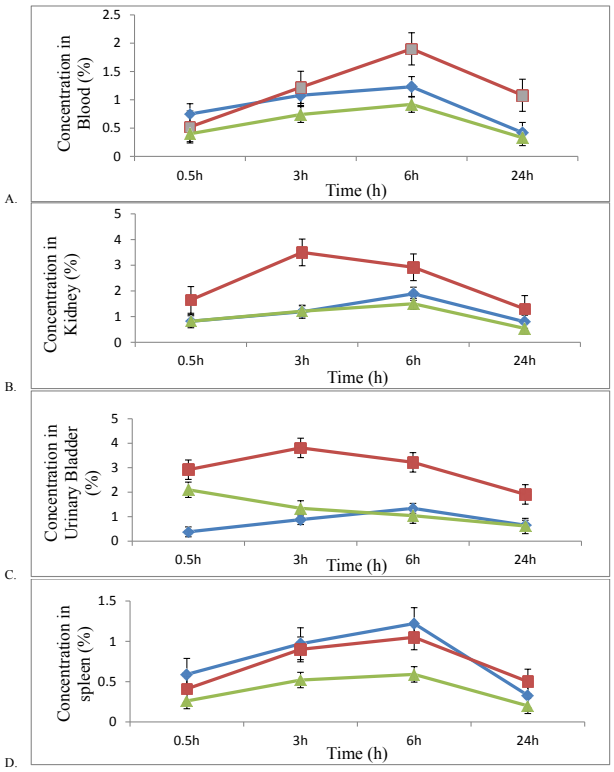

Fig. 4: ^{99m}Tc-P60+ciprofloxacin concentration in various organs at different time intervals

^{99m}Tc-P60+ciprofloxacin concentration in various organs at different time intervals following oral ^{99m}Tc-P60+ciprofloxacin nanoemulsion (—♦—), intravaginal ^{99m}Tc-P60+ciprofloxacin nanoemulsion (—■—) and intravaginal ^{99m}Tc-P60+ciprofloxacin aqueous form (—▲—) administration. A: 99mTc-P60+ciprofloxacin concentration in blood; B: ^{99m}Tc-P60+ciprofloxacin concentration in kidney; C: ^{99m}Tc-P60+ciprofloxacin concentration in urinary bladder; D: ^{99m}Tc-P60+ciprofloxacin concentration in spleen

In the present investigations, it was concluded that P60 and CF showed synergistic effect as per the FIC_{index} against *E. coli* and were successfully encapsulated in an o/w nanoemulsion. The drug release from the encapsulated nanoemulsion was estimated in simulated vaginal media using *in vitro* dissolution apparatus and findings demonstrated sustained release for both P60 and CF. The optimized nanoemulsion was tested for its antibacterial action on resistant representative isolates of *E. coli*, *K. pneumonia*, *P. aeruginosa* and *P. mirabilis*, respectively and findings confirmed the potential of nano formulations to be used against ESBL and MBL producing uropathogens. Our findings revealed that radiolabelled P60+CF nanoemulsion penetrated vaginal mucus most rapidly and reached the target organs such as kidney and urinary bladder. The percent per gram of radiolabelled drug reaching to target organs was significantly higher via IVAG route as compared to oral route. The gamma scintigrams also tracked higher distribution of drug via IVAG route as biodistribution findings.

TABLE 5: PHARMACOKINETICS OF ^{99M}TC-P60+CIPROFLOXACIN NANOEMULSION (ORAL AND IVAG) AND ^{99M}TC-CIPROFLOXACIN AQUEOUS FORM (IVAG) AT DIFFERENT TIME INTERVALS IN SPRAGUE DAWLEY RATS

Formulation and route of administration	Organ	C_{max} (%/g)	T_{max} (h)	AUC
Oral ^{99m}Tc-P60+ciprofloxacin nanoemulsion	Blood	1.23	6	20.77
	Kidney	1.89	6	31.54
	Urinary bladder	1.34	6	22.9
	Spleen	1.62	6	19.32
IVAG ^{99m}Tc-P60+ciprofloxacin nanoemulsion	Blood	1.9	6	33.8
	Kidney	3.50*	3	54.28*
	Urinary bladder	3.81*	3	65.85*
	Spleen	1.05	6	18.6
IVAG ^{99m}Tc P60+ciprofloxacin aqueous form	Blood	0.92	6	8.78
	Kidney	1.50	6	25.15
	Urinary bladder	2.10	0.5	23.33
	Spleen	0.59	6	9.805

Each value is the mean±SD of three estimations. Only statistically significant outcomes at P<0.05 have been reported. AUC is area under the curve, P60 is Polyphenon 60, IVAG is intravaginal. Asterisk mark showed significant values

TABLE 6: DRUG TARGETING EFFICIENCY AND DIRECT TARGET ORGAN TRANSPORT FOLLOWING IVAG ADMINISTRATION OF ^{99M}TC-P60+CIPROFLOXACIN NANOEMULSION

Formulation and route of administration	Target organ	Drug targeting efficiency (DTE %)	Direct target organ transport (DTP %)
P60+ciprofloxacin nanoemulsion (IVAG)	Kidney	106.29	54.5
P60+ciprofloxacin nanoemulsion (IVAG)	Urinary bladder	175.40	43.416

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Conflict of interest:

The authors declare that this paper content has no conflict of interests.

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