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Attenuation of celecoxib cardiac toxicity using Poly(δ-decalactone) based nanoemulsion via oral route

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ABSTRACT

Celecoxib (CLX), a poorly soluble anti-inflammatory drug, requires administration in higher concentrations to produce therapeutic effects, oftentimes resulting in cardiac toxicity. Therefore, in this study, we employed a nanoemulsion technology to improve the solubility of CLX using poly(δ-decalactone) (PDL) polymer as an oil and mPEG-b-PDL as a surfactant. The nanoemulsion (NE) was successfully prepared via the nanoprecipitation method. In vitro characterization was performed for size, drug release, and stability. In vivo studies were performed to establish anti-inflammatory activity, CLX induced cardiac toxicity, and pharmacokinetic profile of NE, post-oral administration. The globular size of less than 100 nm was obtained in NE with high CLX loading. The in vitro drug release studies suggested ~90% of CLX release from NE within 96 h. A significant anti-inflammatory activity with lowered cardiac marker values was observed for CLX NE compared to a marketed drug formulation. The pharmacokinetic study revealed that the mean retention time of CLX was significantly increased with NE in contrast to the marketed formulation, suggesting the advantage of administering CLX in the form of NE owing to the higher solubility and sustained release pattern. The long-term storage stability study reveals that NE does not show significant changes in terms of size with only a slight decrement in CLX content was observed after 24 months. The obtained results indicate that CLX bioavailability has been considerably improved without being toxic to the heart with the aid of NE and advocate the use of PDL NE for developing oral formulations for poorly soluble drugs.

1. Introduction

Celecoxib (CLX) is a selective COX-2 inhibitor, a nonsteroidal anti-inflammatory drug (NSAID) that is well known for having a low risk of gastrointestinal bleeding (Shin, 2018). It is used to treat the symptoms of different forms of arthritis pain and to lessen precancerous polyps in the colon in people with familial adenomatous polyposis (FAP) (Chen et al., 2015). Pfizer markets it under the trade name CELEBREX®, and the FDA initially approved it in 1998. When given orally to dogs, CLX has poor oral bioavailability (only 30%), due to inadequate absorption owing to its poor water solubility. Moreover, CLX taken with a high-fat meal in humans results in only a minor rise in the area under the curve $(AUC)_{0-\infty}$ (11%), which is not clinically meaningful in terms of safety or efficacy (Paulson et al., 2001). According to the US-FDA, chronic use of CLX (CELEBREX®) may increase the probability of fatal myocardial infarction (MI), stroke, and other serious adverse cardiovascular thrombotic events. When compared to placebo, CELEBREX® 400 mg taken twice daily had a 3.4 times (95% confidence interval [CI] 1.4–8.5) higher relative risk for the composite endpoint of cardiovascular death, MI, or stroke; and 2.5 times (95% CI, 1.0–6.4) for CELEBREX® 200 mg taken twice a day (US-FDA, 2008). Long-term oral usage of CLX causes severe adverse effects (cardiotoxic effects and gastrointestinal toxicity), which restricts the drug's administration via the oral route (Salem et al., 2018) . Increasing its aqueous solubility could simultaneously increase its oral bioavailability (Khadka et al., 2014), which could be a potential approach to circumventing these severe side effects owing to high doses.

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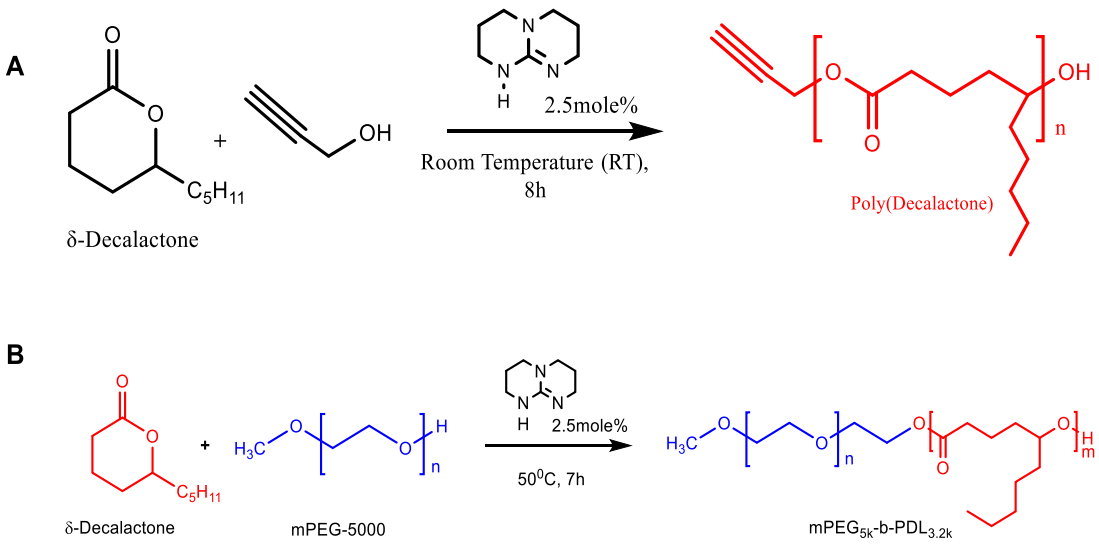

Scheme 1. Synthesis scheme of (A) poly(decalactone) homopolymer and (B) poly(decalactone) block copolymer. The number in subscript denotes the molecular weight of each block(Pyrhönen et al., 2022).

Small dose sizes are required to achieve a sufficient therapeutic response with drugs having high bioavailability and consequently, fewer side effects can be observed (Tamargo et al., 2015).

To achieve this, Qin et al. prepared celecoxib lipid nanoparticles (CXB-NPs) using a film dispersion high-pressure homogenization system. The intragastric (i.g.) delivery of CXB-NPs demonstrated improved bioavailability and prolonged circulation of CLX in cynomolgus monkeys with increased area under the blood concentration (Qin et al., 2023). In another study, Beta-casein (bCN) micelles were utilized to improve the oral bioavailability of CLX. The pharmacokinetics of micelles with 100% CLX encapsulation were tested on pigs and compared with Celebra® (marketed formulation of Pfizer Inc.). The results indicate an increment in AUC and reduction in T_{max} of CLX micelles (Perlstein et al., 2014). PLGA nanoparticles were also investigated to deliver CLX via the oral route with improved digestive tract performance (Li et al., 2022). Alajami et al. used ultrasonic melt emulsification to create CLX-loaded solid lipid nanoparticles (SLN) for oral administration. The authors suggested that CLX-loaded SLN can overcome the common constraint of high burst release and specifically target CLX to the colon when taken orally (Alajami et al., 2022).

CLX was also prepared in nanosuspension form with good colloidal stability using a modified nanoprecipitation method. The incorporation of surface active lipophiles such as Labrafil 1944 CS (oleolyl macrogol glyceride) along with hydrophilic surfactants successfully nanosized the CLX (Malkani et al., 2014). Margulis et al. prepared CLX nanoparticles using solvent extraction from microemulsions in supercritical carbon dioxide with the aid of poly-lactide-co-glycolide and n-butyl acetate to increase its solubility and bioavailability (Margulis et al., 2015). In a similar study, CLX nanoparticles were prepared as a dry powder from a microemulsion system stabilized by a natural surfactants ammonium glycyrrhizinate and soybean phosphatidylcholine, with a 10-fold increment in dissolution compared to CLX (Margulis-Goshen et al., 2010).

Nanoemulsions are another nanoformulation widely used to improve the delivery of pharmaceutically active substances. They are thermodynamically stable, isotropic systems in which an emulsifying agent, such as a surfactant and/or a co-surfactant, is utilized to incorporate two immiscible liquids into a single phase (Aswathanarayan and Vittal, 2019). The dispersed phase (oil) droplet diameter of nanoemulsions created for drug delivery typically ranges from 50 to 500 nm. Nanoemulsions offer greater stability against flocculation, creaming, and sedimentation than traditional emulsions because of the smaller droplet size (Patel et al., 2018; Pathak et al., 2018). Numerous nanoemulsion formulations, including Ropion, Vitalipid, Neoral, Restasis, Limethason, and Diprivan, are already available on the market owing to their benefits.

Nanoemulsions of CLX have also been attempted to improve its solubility and consequently bioavailability. For instance, Shakeel et al. formulated a CLX nanoemulsion using the spontaneous emulsification method, and CLX-loaded SLN using the nanoprecipitation method, as well as CLX solid dispersion using the solvent evaporation approach. The results showed that the nanoemulsion approach yielded the maximum solubility (228.24 mg/mL) and the highest dissolution (99.9%) of CLX. In comparison to SLN and solid dispersion, the results of this investigation indicate that nanoemulsions are promising formulations for improving the solubility and dissolution of poorly soluble drugs such as CLX. Furthermore, the best sustained release profile from nanoemulsions suggests that they can be used for regulated and sustained drug delivery (Shakeel and Faisal, 2010). Using a microfluidizer, Janjic et al. developed a nanoemulsion loaded with CLX and a near-infrared dye. A single I.V. dose of CLX nanoemulsion was found to be capable of reducing inflammation, COX-2 and Prostaglandin E2 level (Janjic et al., 2018; Liu et al., 2020). In another study, Schmied et al. prepared solid self-nanoemulsifying drug delivery systems (S-SNEDDS) by co-extrusion of liquid SNEDDS (L-SNEDDS) and polymeric carrier materials via the HME (hot melt extrusion) method for improving the solubility of CLX and other drugs (Schmied et al., 2022).

However, despite the numerous benefits, the conventional nanoemulsions prepared with oil exhibit some limitations, such as their inability to dissolve high melting point drugs, tedious and expensive fabrication techniques, poor stability due to Ostwald ripening, and the necessity of a large quantity of surfactant, impeding their widespread commercial applications (Wik et al., 2020). To circumvent the drawbacks associated with traditional nanoemulsions, we recently reported a polymer poly(δ-decalactone) (PDL) based nanoemulsion, which was found to be capable of increasing the aqueous solubility of hydrophobic drugs with good stability. In our previous study, we managed to enhance the solubility of CLX by 430-fold (Pyrhönen et al., 2022). Considering this exciting result, we decided to extend the evaluation of CLX nanoemulsion to in vivo for understanding its capability in reducing inflammation and cardiac toxicity.

Therefore, in this study, we prepared a nanoemulsion of CLX using PDL as oil phase and $mPEG_{5k}$-b-$PDL_{3.2k}$ as surfactant by nanoprecipitation method and evaluated this system for drug release, stability and in vivo anti-inflammatory activity on Swiss albino mice via the oral route. The results were compared with free CLX and the marketed

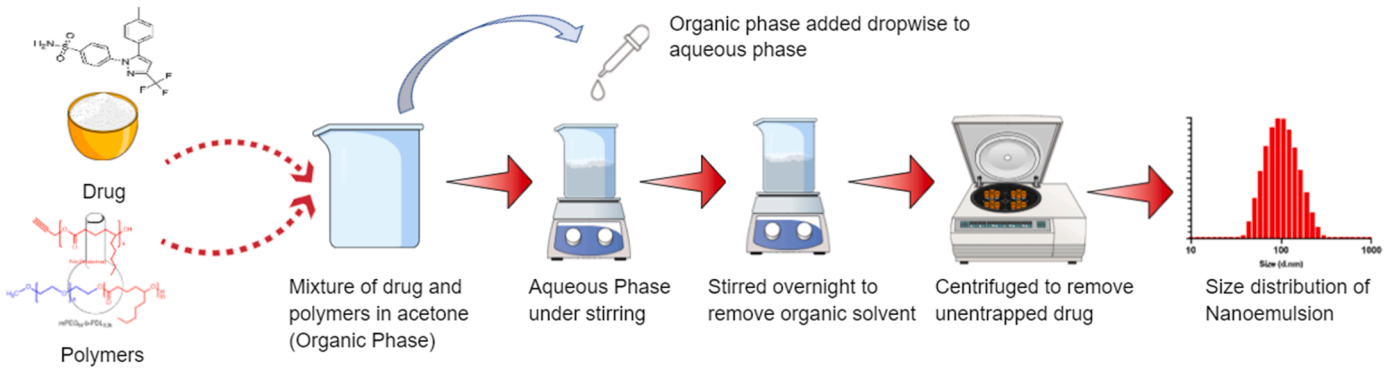

Fig. 1. Schematic representation of nanoprecipitation method for nanoemulsion preparation.

capsules of CLX to ascertain the advantage of the nanoemulsion approach for delivering CLX.

2. Materials and methods

Poly(ethylene glycol) methyl ether (mPEG, Mn = 5.0 KDa), δ-decalactone (≥98%), 1,5,7-triazabicyclo[4.4.0]dec-5-ene (TBD) (98%), propargyl alcohol (99%) were purchased from Sigma Aldrich. Celecoxib (>99%) was purchased from LC Laboratories, Woburn, USA. Methanol for HPLC (≥99.9%), acetone for HPLC (≥99.8%), lambda carrageenan, and carboxy methyl cellulose were purchased from Sigma-Aldrich. $mPEG_{5k}$-b-$PDL_{3.2k}$ and PDL synthesis were already described in our previous publication, and we used the same materials for this study (Pyrhönen et al., 2022). The schematic representation for the synthesis of the polymer is given in Scheme 1. CLX (>99%) was purchased from LC Laboratories, Woburn, USA. Carrageenan was purchased from Sigma Aldrich. Milli-Q water was used for formulation preparation (Milli-Q Synthesis, Millipore, Molsheim, France). The 0.2 μm polyethersulfone membrane filters and 0.45 μm polypropylene membrane filter were purchased from VWR (Puerto Rico and China). Diethyl ether, Hematoxylin & Eosin staining, formalin, Methanol, Sodium Hydroxide (NaOH), Potassium dihydrogen phosphate (KH_2PO_4), Hydrochloric Acid (HCl) were purchased from Sigma Aldrich.

2.1. Nanoemulsion preparation and characterization

The polymer-based nanoemulsion was prepared by a low-energy emulsification method called nanoprecipitation (also known as spontaneous emulsification) using PDL as an oil phase and $mPEG_{5k}$-b-$PDL_{3.2k}$ as surfactant. Briefly, CLX (100 mg), PDL (250 mg), and surfactant (750 mg) were dissolved in acetone (15 ml) with the aid of vortex. This organic mixture was then added dropwise into milli-Q water (50 ml) under stirring and left stirred for at least 12 h at room temperature to ensure the complete removal of the organic solvent (acetone). To separate the unentrapped drug, the nanoemulsion was centrifuged (Microcentrifuge, Scanspeed, Labogene, Lynge, Denmark) for 10 min at 13,500 RPM. The supernatant was then filtered through a 0.45 μm polypropylene membrane filter (Fig. 1).

The drug content was determined using UV–Vis spectrophotometer (NanoDrop 2000c spectrophotometer from Thermo Fisher Scientific) after appropriate dilutions in methanol at λ_{max} – 252 nm. The size and polydispersity index of the nanoemulsion were obtained using dynamic light scattering (DLS) on ZetaSizer Nano-ZS (Malvern Instruments, UK) at 25 °C. Samples were placed into the appropriate cuvette for analysis after being diluted (100 times) with MilliQ water.

2.2. Nanoemulsion stability study

Since the nanoemulsions are thermodynamically unstable and normally stored in liquid form, estimating their stability is a crucial variable (Romes et al., 2021). The filtered samples were kept at cool temperature (4 °C) for 24 months to conduct long-term stability experiments. Initially on day 0 (preparation day) and after 24 months, samples were examined visually for changes in appearance, separation, and creaming. The stability of the formulated nanoemulsion was first assessed by high-speed centrifugation for 30 min at 13,500 RPM. Later, samples were analyzed by DLS and UV–Vis spectroscopy after sonication for 5 min, to observe the change in size and drug content, respectively.

2.3. Drug release

The release profile of CLX from nanoemulsion was determined by a dialysis method at 37 °C (Bansal et al., 2018). Briefly, a calculated amount of CLX loaded nanoemulsion equivalent to 40 μg of CLX was diluted up to 2 mL in simulated gastric fluid (SGF, pH 1.2). The nanoemulsion solution was then placed in dialysis tubing (Float-A-Lyzer) having the molecular weight cut-off (mwco) of 3.5–5 kDa. The sample was then dialyzed against 500 mL of SGF (pH-1.2) for 6 h and then the release media was replaced with SIF (pH 6.8). The release media was replaced with fresh media (SIF) in every 24 h. The volume of solution in the dialysis tubing were measured at appropriate time intervals (~6 h) and restored to the original level with the respective solvent, if necessary. Samples (1 ml) were withdrawn directly from the dialysis tubing (after shaking) at predetermined time intervals and were analyzed with UV- Vis spectrophotometer to calculate the amount of CLX remaining in the nanoemulsion. The analyzed samples were transferred back to dialysis tubes to maintain the original volume. For analyzing the release pattern of free drug, the CLX was dissolved in methanol and the experiment was performed using methanol as release media due to the poor stability of the drug in aqueous medium.

2.4. Anti-inflammatory studies using carrageenan-induced paw edema model

Paw edema was induced (Cunningham and Keaveny, 1978; Larsen, 1972) by injecting 25 μL of 1% w/v lambda carrageenan suspended in 1% carboxy methyl cellulose (CMC) into sub-plantar tissues of the left hind paw of each mouse. Male mice were divided into four groups: carrageenan control group, marketed formulation group (Celebrex 100 mg, Pfizer), pure drug group, and the nanoemulsion group, consisting of six animals. Mice between 8 and 12 weeks of age were housed in a temperature-controlled environment with a 12-h light/dark cycle. All mice received a standard diet and water ad libitum. The animal experiments were approved and compliant with the Institutional Animal Ethics Committee constituted by CPCSEA, Govt. of India (approval number/IAEC/2021/01/03). Paw thickness was measured just before injecting the lambda carrageenan, that is, at "0 h" and then at 0.5, 1, 2, 3, 4, 6, 8, and 24 h using a Vernier Caliper. The anti-inflammatory activity was calculated as percentage inhibition of edema in the animals treated with the formulation/drug under test in comparison to the

carrageenan control group. The pure drug and marketed formulation were suspended in 0.5% CMC in distilled water before administration. The pure drug, marketed formulation, and nanoemulsion formulation were orally administered to mice through 18–20 gauge feeding tubes about 1.5 inches in length, slightly curved arc with a rounded tip at a dose of 40 mg/kg body weight.

The percentage (%) inhibition of edema was calculated using the formula below:

$$\text{Percentage inhibition} = [(T_0\text{--}Tt) / T_0] \times 100$$

Where, Tt is the thickness of the paw of mice administered test samples at the corresponding time and T_0 is the paw thickness of mice of the control group (Carrageenan treated) at the same time.

2.5. Toxicity studies

2.5.1. Biochemical analysis

For biochemical analysis, mice were divided into four groups: carrageenan control group, marketed formulation group (Celebrex 100 mg, Pfizer), pure drug group, and the nanoemulsion group, consisting of six animals each. The dose preparation was done in the same way and treatment was done with the same dose and route as stated above in anti-inflammatory study. Blood samples were collected by retro-orbital sinus puncture in the absence of anticoagulant under light anesthesia induced by diethyl ether post treatment. The serum was collected from clotted blood after centrifugation at 5000 × g for 10 min at 4 °C and used for biochemical analysis using a Biochemistry Analyzer (prietest TOUCH PLUS, Robonik, India). The estimation of the values of key parameters like urea, uric acid, creatinine, creatine kinase (CK-MB), and troponin-I were performed at local pathology.

2.5.2. Histopathological analysis

Post blood collection for biochemical analysis, animals were sacrificed. The heart and kidney were collected and fixed in 10% neutral buffered formalin for histopathological analysis. The fixed specimens of the heart and kidney were processed for dehydration, clearing, and impregnation as per standard protocol (Chevrier et al., 2022). The specimens were embedded in paraffin blocks using an embedding station (Sakura, Japan) and serial sections of 4 μm thickness were cut using a microtome (CUT6062, SLEE Medical GmbH, Germany). The tissue sections were then stained with hematoxylin & eosin staining. The mounted specimens were observed for any pathological changes under light microscopy.

2.6. Pharmacokinetic study

The marketed formulation and nanoemulsion were administered to mice for analysis of pharmacokinetic parameters (Giri et al., 2022). The drug formulations were orally administered to mice (PO, $n = 3$ for each time point) at a dose of 40 mg/kg body weight. Blood samples were collected at 9 time points, i.e., 0, 0.5, 1, 2, 3, 4, 6, 8, 24 h, and plasma was separated. The plasma was then analyzed by HPLC to detect the amount of CLX. Quantification of CLX was conducted using a HPLC system (Giri et al., 2022). The Agilent (1100) HPLC system was used with Agilent ChemStation software, and a C18 (Agilent)-(3), 250 × 4.6 mm (5 μm) column maintained at 25 °C. The mobile phase was composed of a mixture of methanol and 0.05% ortho-phthalaldehyde in a ratio of 75:25 v/v, respectively. The UV spectrum was recorded at 252 nm with a flow rate of 1.0 mL/min and an injection volume of 10 μL.

Pharmacokinetic parameters (C_{max}, T_{max}, AUC, clearance, half-life, mean retention time, etc.) were calculated using PK Solver 2.0 using MS Excel software.

Table 1
Drug content, Z-average size, and PdI of freshly prepared CLX loaded nanoemulsions (0 month) and after 24 months of storage at 4 °C.

Samples	CLX content in mg/ml (±SD)	Z-average size in nm (±SD)	PdI (±SD)
Nanoemulsion (0 month)	1.9 ± 0.06	93.4 ± 0.29	0.138 ±0.01
Nanoemulsion (24 months)	1.6 ± 0.06	105.6 ± 1.71	0.145 ±0.01
% Change from 0 month to 24 months	−15.5%	+13.1%	+5.1%

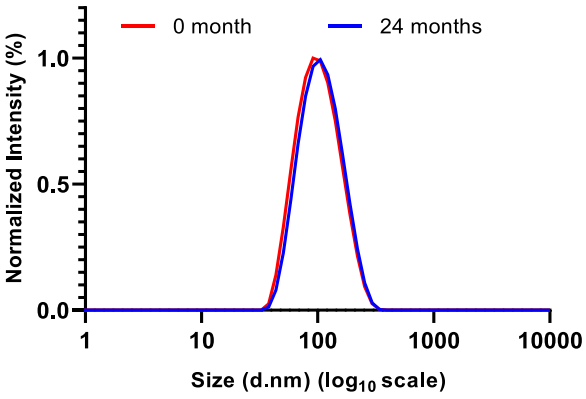

Fig. 2. Hydrodynamic size distribution by intensity of CLX loaded nanoemulsion at time 0 month and 24 months, when stored at 4 °C.

2.7. Statistical analysis

All values were expressed as the mean ± standard error of the mean (mean ± SEM) of six/three independent experiments. The data for multiple treatment groups were analyzed using one-way ANOVA (Multiple Comparisons) followed by Tukey's multiple comparisons test, computed using GraphPad Prism (Version 5.02) software, if not stated distinctly. Differences were considered as statistically significant at $P < 0.05$, when compared with controls.

3. Results and discussion

3.1. Preparation and characterization of nanoemulsion

Following our previous study, the PDL based nanoemulsion was prepared with the aid of mPEG-b-PDL as surfactant through a low-energy nanoprecipitation process. Due to the improved compatibility of mPEG-b-PDL with PDL, as observed in our earlier investigation, the 1:3 ratio of polymer to surfactant produced a stable emulsion (Pyrhönen et al., 2022). No CLX precipitation was visually observed after the evaporation of organic solvent during nanoemulsion preparation, suggesting near 100% encapsulation of drug. However, to make sure that the nanoemulsion is free from unentrapped drug, it was purified by centrifugation followed by filtration. Due to the poor aqueous solubility of CLX, any unentrapped drug must be removed from the formulation upon filtration. The purified nanoemulsion was then characterized for its drug content and size after appropriate dilutions. The results are shown in Table 1.

In general, nanoemulsions are more stable than conventional emulsions due to the small droplet size, thus circumventing phase separation, coalescence, and/or flocculation. However, the shelf life of the nanoemulsion depends on droplet size, polydispersity and type of oil used (Bernardi et al., 2011; Liu et al., 2019). Since we have used liquid polymer instead of oil in the preparation of our nanoemulsion, we

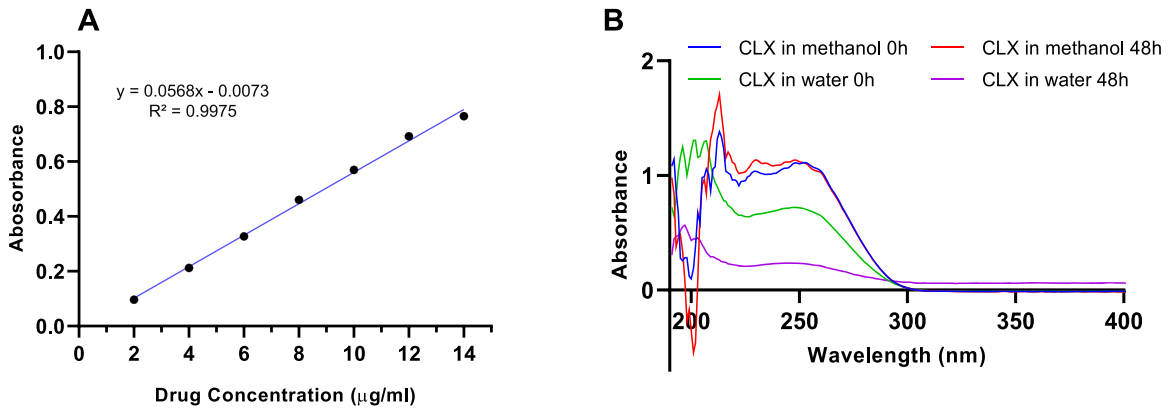

Fig. 3. A) calibration curve of CLX in methanol and (B) UV spectra of CLX solution in methanol and in water prepared using CLX stock in methanol. The absorbance spectra indicate a decrease in CLX concentration in water and no change in CLX concentration in methanol from time 0 h to 48 h at 252 nm when stored at 37 °C.

assumed that the process of Ostwald ripening (destabilization process of nanoemulsion) had been significantly slowed down (Pyrhönen et al., 2022; Wik et al., 2020). Thus, to analyze the stability of the nanoemulsion as well as the loaded CLX upon storage, it was stored for 24 months at 4 °C. No evidence of visible changes in the emulsion was observed after 24 months of storage. To observe the change in size and PdI, the nanoemulsion was analyzed with DLS. Although the nanoemulsion showed 13% increment in the Z-average size and 5% increment in PdI, the size was still close to 100 nm (Fig. 2, Table 1).

Furthermore, the change in CLX content in the nanoemulsion was evaluated with the aid of UV spectrophotometry. To determine whether the UV method is efficient in determining the degradation of CLX, we did some control measurements. The stock solution of CLX in methanol was diluted with water (20 μg/mL) and with methanol (20 μg/mL), after which the absorbance was measured at time 0 h. The samples were then stored at 37 °C and the changes in absorbance in CLX solution were analysed. As shown in Fig. 3, there was a clear reduction in absorbance after 48 h of storage of CLX in water; on the other hand, CLX in methanol showed almost similar absorbance, indicating the adequacy of this method to reveal CLX degradation.

For CLX in nanoemulsions, a small apparent difference in drug concentration (15.5% decrement) was observed after 24 months of storage at 4 °C compared to the initial value (Table 1). Usually, the expiration date of any medicament represents a 10% degradation of its active content (API) from the initial value. Considering the poor aqueous solubility and stability of CLX, the current finding suggests that the nanoemulsion offers good stability and the capacity to prevent significant drug degradation for at least 24 months, when stored at 4 °C. However, further stability studies are warranted according to ICH guidelines to establish the stability of the formulation and shelf life.

3.2. *In-vitro release study*

For any nanotechnology-based formulation, it is important that the encapsulated drug will release at a sufficient rate to produce a pharmacological response. The control sample containing the pure drug (CLX in methanol) showed almost 100% release within 24 h. To assess the release pattern of CLX from the nanoemulsion, we performed experiments at two different pH. Since the prepared nanoemulsion was aimed at oral delivery of CLX, SGF was also chosen in addition to SIF as the release media to monitor the release pattern of CLX for 96 h. To mimick the oral delivery and assuming 6 h as gastric residence time for nanoemulsion, SGF was choosen as release media for first 6 h and later replaced with SIF. An initial burst release of CLX was observed from the nanoemulsion followed by a slow-release pattern upto 96 h. In the first 6 h, 45.0% of CLX was released at pH 1.2. The release of almost 45% of drugs in the beginning could be beneficial in achieving the minimum

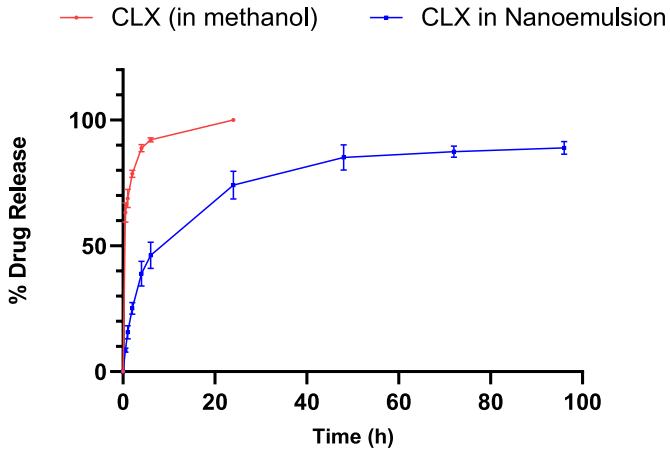

Fig. 4. In-vitro CLX release (%) from the nanoemulsion, in which nanoemulsion was incubated first in SGF (pH 1.2) for an initial 6 h and then subsequently in SIF (pH 6.8) buffer at 37 °C ($n = 3$). The release pattern of free CLX was observed by preparing CLX sample in methanol.

effective concentration of CLX in the blood to produce the therapeutic effect. After 96 h, the cumulative amount of CLX release was 90.0%. We do not observed a notable increment in the release percentage of CLX past 48 h in SIF after which a platau was attained (Fig. 4). However, contrary to the expectation owing to the amorphous nature of PDL polymer, we did not observed complete release of CLX from the nanoemulsion within 96 h. This phenomenon could be due to the anticipated equilibration of CLX in empty mPEG-b-PDL micelles (surfactant) and nanoemulsions within a closed chamber (dialysis tubing). The reversible drug binding to empty mPEG-b-PDL micelles was also observed in a previous study and was found to be responsible for retarding the release rate (Bansal et al., 2015).

3.3. *In vivo studies*

3.3.1. *Anti-inflammatory studies by carrageenan-induced paw edema model*

All the animals were treated with the same batch of nanoemulsion for in vivo studies. Carrageenan-induced paw edema model was used to assess the comparative anti-inflammatory activity of the pure drug, a marketed formulation, and CLX loaded nanoemulsion with the control group (only carrageenan). The results revealed that all groups inhibited the inflammation in the study model, although the level of significance was considerably different. The nanoemulsion is more efficacious with higher level of significance ($P<0.001$) compared to pure drug ($P<0.05$)

Table 2
Percentage inflammation inhibition by different formulations compared to positive control group ($n = 6$).

Time (h)	Pure Drug (%) (±SD)	Marketed formulation (%) (±SD)	Nanoemulsion (%) (±SD)
2	5.4 ± 0.00683	8.8 ± 0.002236	12.3 ± 0.005163
4	18.1 ± 0.004772	20.7 ± 0.004215	28.1 ± 0.004943
6	19.3 ± 0.004281	23.1 ± 0.005425	32.9 ± 0.004281
8	25.0 ± 0.003415	28.3 ± 0.005163	35.9 ± 0.004281
24	24.5 ± 0.004281	28.7 ± 0.005626	35.5 ± 0.004215

and marketed formulations ($P<0.05$). The percent inhibition compared to control group by pure drug, marketed formulation, and nanoemulsion at different time points is presented in Table 2. The effect sustains for 24 h in similar magnitude, i.e., 24.47%, 28.72%, and 35.46%, respectively. The results clearly indicate that the nanoemulsion group is showing a considerably higher magnitude of anti-inflammatory activity compared to pure drug and marketed formulation (Fig. 5).

Considering the% inhibition of inflammation and reduction in mouse paw thickness, it can be concluded that the nanoemulsions demonstrated significant activity of CLX compared to the other tested groups. The less effective response of pure drug and marketed formulations compared to nanoemulsions suggested the importance of delivering drug using nanotechnology, which is known to change the pharmacokinetics of the drugs (Mitchell et al., 2021). The nanoemulsion formulation has subsided the inflammation to a greater extent and proved to be present in a clinically significant doses to exhibit its efficacy.

3.3.2. *Toxicity studies*

CLX is known to have cardiac toxicity at high dose (Ahmad et al., 2018; Engle et al., 2009). The 40 mg/kg body weight doses of the drug formulations were administered to mice orally to see any possible toxic effect of CLX in the different formulations. To evaluate the cardiac and renal toxicity, cardiac biomarkers such as Cardiac Troponin I and CK-MB as well as renal biomarkers such as Urea, Uric Acid, and Creatinine were analyzed. The cardiac biomarker levels were found to be significantly higher compared to the control group for the marketed formulation group, i.e., Troponin I ($P<0.0022$) and CK-MB ($P<0.0001$) (Fig. 6), which indicates severe cardiac toxicity. The remaining two groups, i.e., pure drug and nanoemulsion, were found to be in the normal range and no statistically significant differences were observed. The finding suggests that the marketed formulation is adversely affecting the cardiac tissues and thus, causing cardiac damage that leads to an increase in Troponin I and CK-MB levels in the blood (Fig. 6). In comparison of renal biomarker values of the control group with treatment groups, i.e., pure drug, marketed formulation, and nanoemulsions, no significant variations (level considered significant at $P>0.05$) in the parameters were observed (Fig. 7). The absence of cardiac toxicity with the pure drug could be due to its poor aqueous solubility resulting in poor bioavailability. At a subclinical dose, a drug will show limited efficacy and toxicity as its efficacy and toxicity are both dependent on bioavailable dose. In contrast, the marketed formulation may have several additives that aid in increasing its solubility to an extent that it becomes toxic to the cardiac tissues.

To further confirm the toxicity in cardiac and renal tissues, histopathological studies were performed. The heart and kidney tissues were harvested from the animals and microscopic studies were performed on histological slides. To observe kidney tissue damage, microscopic studies of kidney tissue were performed to detect any sort of abnormality in the cortex, medulla, renal papilla & renal pelvis in kidney. Kidney was

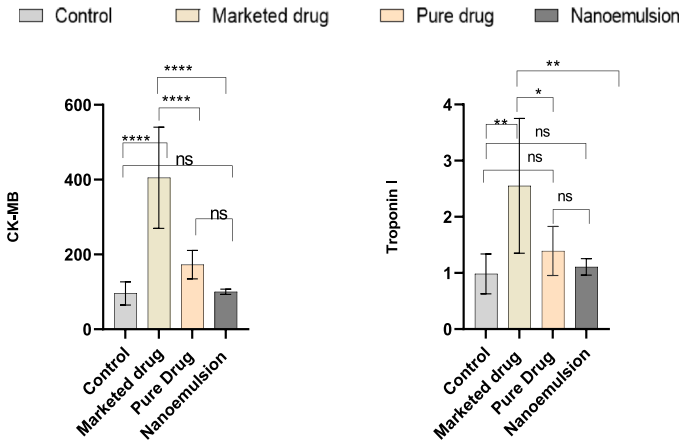

Fig. 6. Cardiac markers CK-MB (U/L) and Troponin I (ng/mL) level in the samples collected from animals ($n = 6$) after treatment with water (vehicle only, control group), nanoemulsion, and marketed drugs to estimate the cardiac toxicity. A significant difference in levels was observed for the marketed drugs compared to the control group and nanoemulsions ($P<0.05$) (ns= non significant).

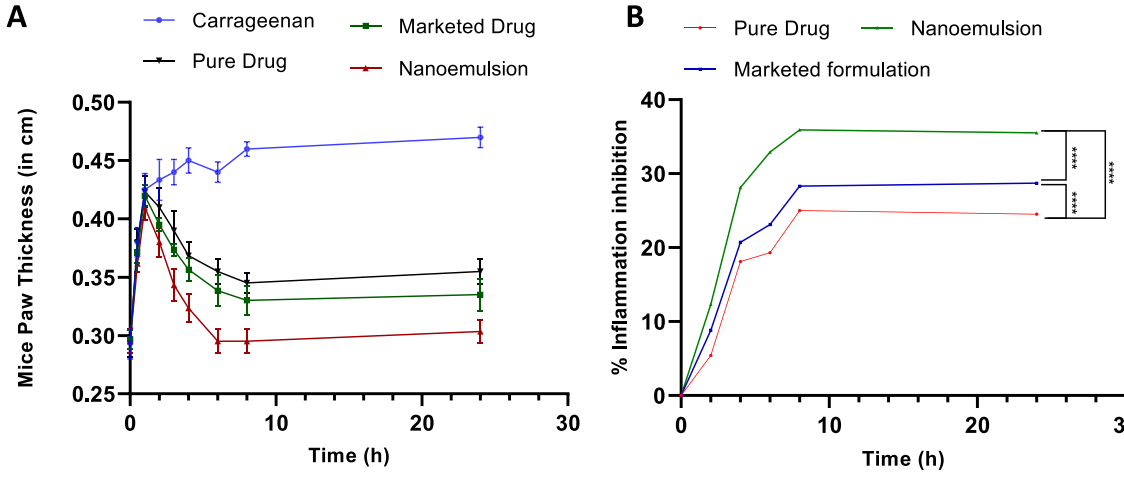

Fig. 5. (A). Anti-inflammatory activity of nanoemulsions, marketed drugs and pure drugs after oral administration to mice ($n = 6$) presented as reduction in paw thickness. The standard carrageenan induced mouse paw edema model was used in this study. (B) Representation of anti-inflammatory results in terms of% inflammation inhibition compared to control group (carrageenan). The data of multiple treatment groups were analyzed using two-way ANOVA (Multiple Comparisons) followed by Tukey's multiple comparisons test, computed using GraphPad Prism (Version 5.02) software, which shows higher level of significance ($P<0.0001$).

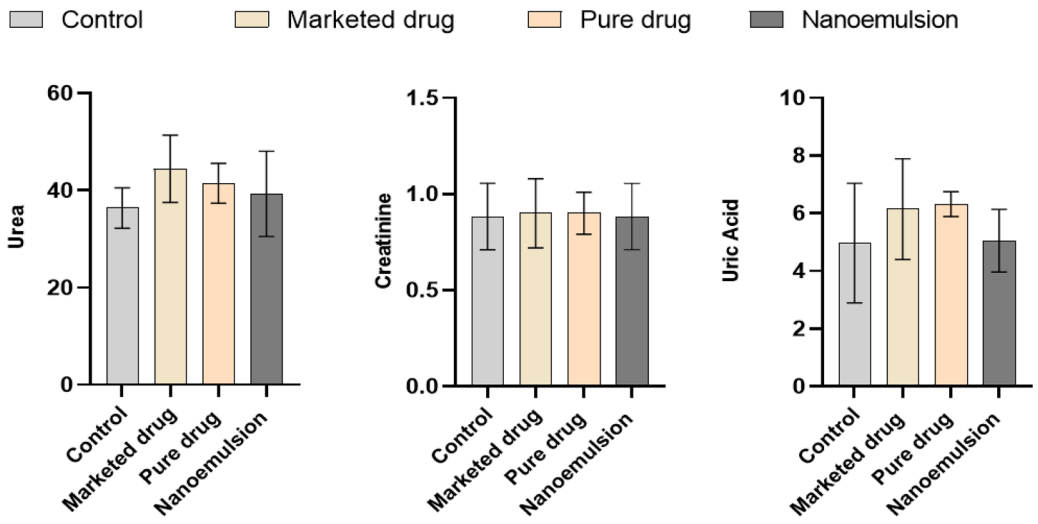

Fig. 7. Estimation of renal toxicity of pure drugs, nanoemulsion, and marketed drugs by comparing the renal markers urea (mg/dl), creatinine (mg/dl) and Uric Acid (mg/dl) levels with the control group. No statistically significant difference was found in any group ($n = 6$).

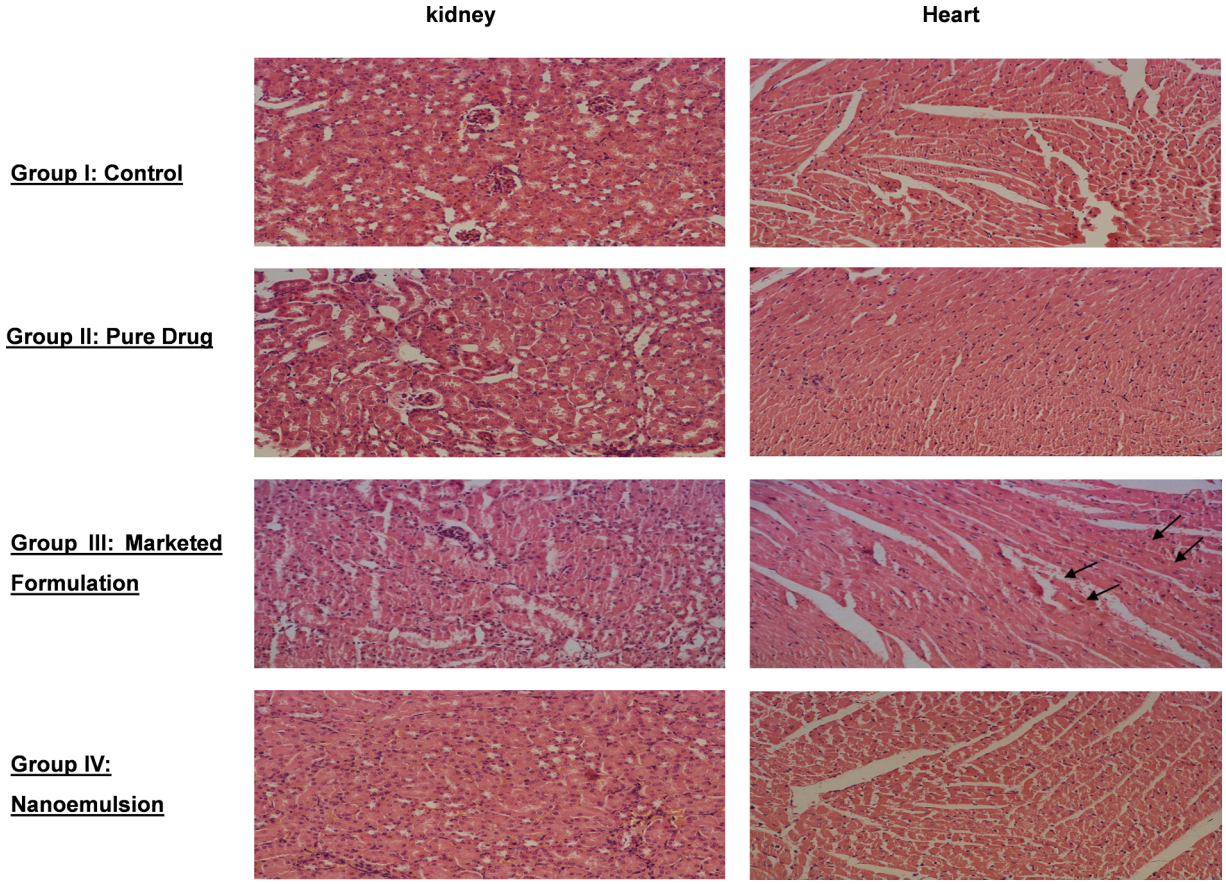

Fig. 8. Histopathological evaluation of major organs of the mice treated with different samples (40 mg/kg, PO). The black arrows show abrasion where the rough edges of the cells indicate damage.

observed for the shape & size of glomeruli, renal tubules & collecting duct. They were also observed for any pathological changes such as renal tubule dilation, inflammatory cell infiltration, cytoplasmic degeneration, hyperplasia and atrophy, hypertrophy of renal tubules, fatty degeneration, mineralisation and necrosis (H&E, 20x). The microscopic studies revealed no damage to renal tissues after treatment (Fig. 8). To observe heart tissue damage, microscopic studies of heart tissue were observed for any sort of abnormality in the ventricles & atrium of heart, size & shape of myocardium, endocardium and epicardium appearance. Heart was also observed for any pathological changes such as inflammatory cell infiltration, atrophy, hypertrophy of myocardium, fatty degeneration, cytoplasmic degeneration, mineralisation, amyloid degeneration, and necrosis (H&E, 20 x). Hemorrhage (black color arrow, Fig. 8) in the myocardium focal minimal in heart was observed in the marketed formulation group. This is the probable cause of the elevated Troponin I and CK-MB levels in this group. The findings

Table 3
Pharmacokinetic parameters of marketed formulations and nanoemulsions (n = 3).

Parameter	Unit	Marketed Formulation (±SD)	Nanoemulsion (±SD)
$t_{1/2}$ka	h	0.87 ± 0.52	0.89 ± 0.61
$t_{1/2}$k10	h	1.99 ± 1.43	5.79 ± 6.12
V/F	(mg)/(μg/ml)	0.43 ± 0.22	0.64 ± 0.33
CL/F	(mg)/(μg/ml)/h	0.17 ± 0.08	0.11 ± 0.05
T_{max}	h	1.60 ± 0.55	1.96 ± 0.60
C_{max}	μg/ml	1.48 ± 0.17	1.24 ± 0.18
AUC $_{0-t}$	μg/ml*h	7.76 ± 2.94	11.10 ± 4.50
AUC $_{0-\infty}$	μg/ml*h	7.80 ± 2.96	13.20 ± 8.10
AUMC	μg/ml*hˆ2	35.32 ± 22.73	170.06 ± 220.23
MRT	h	4.13 ± 1.82	9.63 ± 7.98

$t_{1/2}$ka = absorption half-life, $t_{1/2}$k10 = terminal half-life, V= apparent volume of distribution, F= fraction of drug absorbed, CL= clearance, t1/2 = half-life, MRT = mean residence time.

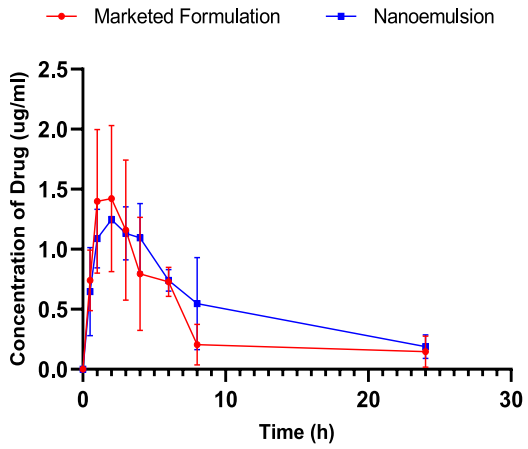

Fig. 9. Plasma drug concentration time profile of the marketed formulation and nanoemulsions after a single PO dose of 40 mg/kg (n = 3).

indicate possible toxicity of the marketed formulation and confirm the biochemistry results.

Remarkably, at the same dose, even if the CLX in nanoemulsion was present in the circulation at a clinically significant dose, it did not show any toxicity, which makes it superior to marketed drug. The superiority of nanoemulsion formulation could be attributed to the controlled release pattern of the drug from the formulation.

3.3.3. Pharmacokinetic studies

To better understand the pharmacological activity with reduced toxicity of the nanoemulsion, pharmacokinetic studies were performed. The nanoemulsion and marketed formulation were administered to mice to estimate various pharmacokinetic parameters, including maximum plasma concentration (C_{max}), time to attain maximum plasma concentration (T_{max}), and bioavailability (AUC) of the CLX administered in different forms. The free drug was excluded from this study as its performance was poorer among all groups.

The study revealed an enhanced bioavailability of the CLX from nanoemulsions compared to the marketed formulations (Table 3). It was found that the Mean Retention Time of the drug has considerably increased for the nanoemulsion compared to the marketed formulation from 4.13 h to 9.63 h. Compared to the marketed formulations, there was a slight increase in the average values of area under the curve from 0 to time t (AUC $_{0-t}$), area under the curve from 0 to time infinity (AUC $_{0-\infty}$), and area under the moment curve (AUMC) (Fig. 9, Table 3). The data indicates that the bioavailability of CLX from the nanoemulsion has not been compromised after processing into polymeric nanoemulsion, indicating the formulation capability to provide similar or better anti-inflammatory activity without cardiac toxicity. The average time to reach the maximum plasma concentration of the drug through marketed formulation and nanoemulsion was found to be 1.6 h and 1.96 h, respectively. The data indicates that CLX is taking longer time to reach its peak concentration level in nanoemulsions compared to the marketed formulations. Furthermore, comparing mean values, the maximum plasma concentration (C_{max}) of the drug achieved through the marketed formulation and nanoemulsions was found to be 1.48 μg/ml and 1.24 μg/ml, respectively. This clearly indicates that the C_{max} achieved post-marketed formulation administration was higher, i.e., 19.35% compared to the C_{max} achieved post nanoemulsion administration. The higher C_{max} achieved from marketed formulation may be contributing to its severe cardiac toxicity as the toxic level of the drug (higher concentration in plasma) might be attained from the marketed formulation. The optimum plasma level peak of CLX with optimum plasma concentration might have prevented the possible cardiac toxicity from nanoemulsion due to the slow release of CLX. The pharmacokinetic studies reveal that the marketed formulation shows a rapid release of drug leading to a high peak of the drug level along with a rapid decline of its plasma concentration, probably leading to toxicity and relatively less efficacy compared to the nanoemulsion.

4. Conclusions

In this study, we successfully developed a CLX-loaded nanoemulsion with the aid of polymer PDL instead of conventional oil, stabilized by mPEG-b-PDL block copolymer as surfactant. The stability study suggested that the nanoemulsion are stable as the size and drug content values was only slightly deviated from the initial values upon 24 months of storage. As per the in vitro release study, up to 90% of CLX was released in the simulated GIT media within 96 h from nanoemulsion suggesting sustained release behavior along with intial burst release. Although, all groups tested for anti-inflammatory activity showed activity, the nanoemulsion group showed a considerably higher level of anti-inflammatory activity. Moreover, the nanoemulsion group confirmed its safety in terms of cardiac toxicity caused by CLX owing to the sustained release behavior, which resulted in slightly lower plasma peak concentration compared with the marketed group. The presented study reveals the advantage of using PDL polymer based nanoemulsion to deliver poorly water-soluble drugs with decreased cardiac toxicity without compromising the activity via the oral route.

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Author contributions

Conceptualization, K.K.B; methodology, J.V. and S.M.; formal analysis, J.V. and S.M.; investigation, K.K.B.; resources, C.E.W. and J.M.R; writing—original draft preparation, J.V. and S.M.; writing—review and editing, K.K.B. and J.M.R.; supervision, C.E.W. and J.M.R.; project administration, K.K.B.; funding acquisition, J.M.R. All authors have read and agreed to the published version of the manuscript.

Declaration of Competing Interest

The authors declare no conflicts of interest.

Data availability

Data will be made available on request.

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