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Preparation and Characterization of Berberine-loaded Dextrose-modified Nanostructured Lipid Carriers

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ABSTRACT: Rheumatoid arthritis (RA) is an autoimmune ailment that manifests rapidly in troublesome locomotive activity and often restricted movements in patients. The conventional therapy for RA is done through NSAIDs but often showed unsatisfactory therapeutic outcomes. Recently nature derived anti-arthritic phytoconstituents have been explored for RA therapy. Berberine (Br) has attained considerable attention as a natural xenobiotic for RA therapy. However, its use is restricted due to extensive first-pass elimination, low aqueous solubility, low permeability, and low oral bioavailability. Lipodal drug delivery systems can address these challenges thus in the present research, we have formulated dextrose (DEX) modified Br-loaded Nanostructured Lipid Carriers (NLCs) employing the melt-emulsification method. The prepared DEX-Br-NLCs were assessed for percent entrapment, drug loading, in-vitro release and cytotoxicity on HEK-293T cells. The prepared DEX-Br-NLCs exhibited a particle size of 200.9±0.53 nm, percent entrapment of 68.42±1.51%, drug loading of 3.58±0.14% and 80.32±6.65% drug release respectively over a period of 24 hr. Internalization of Rhodamine loaded DEX-Br- NLCs was confirmed via cellular uptake study. Cytotoxicity studies revealed that the prepared formulation was non-toxic. Thus DEX-Br-NLCs were successfully prepared, which might further improve the oral bioavailability, solubility and permeability of Br and produced controlled release of the drug localized inflammation.

Keywords: Berberine, Rheumatoid arthritis, NLCs, Complete Freund's adjuvant, Auto-immune.

INTRODUCTION

Rheumatoid arthritis (RA) is distinguished by significant joint and bone destruction caused by an increased autoimmune response at the articular sites. RA affects 3 people out of every 10,000 people worldwide each year, or 1% of the population. Early signs include morning stiffness, swollen and painful joints, and general malaise (Gravallese & Firestein 2023). Chronic RA, if left untreated, can develop systemic inflammation, abnormalities of the heart, liver, gut, and muscles, as well as, in some cases, cognitive deterioration (Aletaha & Smolen 2018). It typically begins between 20 and 40 years of age, and its prevalence rate ranges from 0.3 to 1%. The development of this disease is known to be influenced by a number of factors. About 60% of cases of RA are associated with genetic predisposing factors, which are heightened by environmental variables (Sayah & English 2005). Various synthetic drugs, herbal remedies, and biological therapies are now available as RA treatments. Potential delivery methods for RA management are based on conventional treatment but they still carry significant threats of toxic and therapeutic intolerance consequences brought due by higher dosage (Yang *et al.*, 2013). Patients do require cutting-edge treatments with few adverse effects. Nanoparticles are the modern delivery system of drugs that can be used to target pharmaceuticals to a specific location in cells and tissues. They also have a higher bioavailability, improved pharmacokinetics profile, and a rise in the loading of drug molecules which allows an effective and safe way of delivering the drug molecule, hence helping in the treatment of diseases (Dolati *et al.*, 2016). Nanoparticles loaded with the drug have numerous advantages over conventional medications, such as improved solubility of drugs, targeted remembrance of the cells to be targeted, fewer systemic side effects, prevention from the degradation of drug, provide release of drug in a controlled manner, use of diagnostic tools as theranostic agents and elevation of drug transport across the biomembrane (Li *et al.*, 2019). The more recent approach to treating RA, known as the "treat-to-target" technique, involves careful monitoring of the disease's course and management if the appropriate medicine is not effective (Mandl & Aletaha 2019). At the present time, RA treatment focuses on minimising disease activity followed by a potential remission preventing joint deformities and disease progression. The active targeting is accomplished by surface modification of nanoparticles, which enhances entry via interaction of the targeting module with the

overexpressed components of inflammatory cells (Elkomy *et al.,* 2022). Surface modification employs specific ligands as a result of interaction bonding with overexpressed components in inflammatory cells, including moieties such as proteins, carbohydrates, peptides, and antibodies (Guerrini *et al.,* 2018). Lectins can be used in RA therapy to interact with cells. These lectins are useful as targeting moieties because they recognise carbohydrate moieties including dextrose, mannose, glucose, and their derivatives (Jack *et al.,* 2001). For the treatment of RA, herbal remedies have been utilised for a very long time as alternative methods of therapy. Because they often have fewer negative effects and lesser toxicities, these are currently capturing the interest of researchers who investigate plant extracts and herbal formulas. As a result, the use of medicinal plants in seek for novel medications is becoming more and more important (Yang *et al.,* 2013). Many Chinese plants such as Cortex phellodendri, Mahonia bealei, and Rhizomacoptid possess therapeutic potentials as per reported literature. These plants contain berberine (Br), a bisbenzylisoquinoline alkaloid (Gravallese & Firestein 2023). It has been used for eras to cure a lot of diseases due to its anti-inflammatory (Song *et al.,* 2020) and antibacterial characteristics. Furthermore, rat chondrocytes activated by sodium nitroprusside and the proliferation of arthritic rat cartilage tissue are both promoted by Br. Hu et al. demonstrated that Br possibly will decrease immunological responses, promote dendritic cell death in vitro, and alleviate collagen-induced arthritis in vivo (Hu *et al.,* 2011). Numerous studies have shown the anti-arthritic activity of Br (Zhou *et al.,* 2019). By activating AMPK in macrophages, Br can reduce the production of TNF-α, IL-1b, IL-6, IL-17 inducible NOS (iNOS), monocyte chemoattractant protein 1 (MCP-1), cyclooxygenase-2 (COX-2), and matrix metalloprotease 9 (MMP9) and increase IL-10 and TGF-b. Br also functions as a strong activator of miR-23a, which inhibits downstream activity of kinases like GSK-3b and inflammatory transcription factors like ASK1 in RA (Huang *et al.,* 2021). The low oral bioavailability of Br is attributable to its first-pass elimination in both hepatic and intestinal organs; the self-aggregation property of Br, which makes it less soluble in the alimentary tract; Br possesses less permeableness through the mucosa of the intestine; and the fact that Br is a P-gp substrate, that slows its transportation via gut (Huang *et al.,* 2021). All the above-mentioned drawbacks limit the use of Br for oral administration and clinical use.

In the current work, we reported Br-NLCs modified with dextrose (DEX) for administration to the RA inflammatory site. DEX was employed as a ligand for selective delivery to carbohydrate-specific lectin receptors. The polymeric structure of DEX improves surface functioning by accumulating and retaining carbohydrate-coated NLCs as a result of lectin-carbohydrate interaction. The carbohydrate-modified system promotes lectin-carbohydrate interaction, which can lead to increased accumulation and retention in the targeted organ. These surface modified carriers connect to overexpressed lectin receptors with high affinity due to the cluster effect. The current research work foresees overcoming the complications of Br which are poor oral bioavailability and water solubility through the formulation of surface-decorated Br-NLCs, which may improve its oral bioavailability and reduce dose and dose-related adverse effects.

MATERIAL AND METHOD

A. Materials

Drug and Chemicals. Berberine Chloride, Oleic acid, Capric acid, MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide), Rhodamine B, DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride), Dextrose and Chitosan were procured from M/s Sigma Aldrich, USA. Phospholipid GmbH (Nattermannallee, Germany) gifted a sample of Phospholipon® 90 G. Glyceryl Di-stearate, and Triethanolamine were acquired from Loba Chemi Pvt. Ltd, Mumbai. DMSO (Dimethyl sulphoxide), Sodium Hydroxide, and Potassium Hydroxide were purchased from SD Fine Chem Pvt. Ltd. Mumbai. All other analytical grade chemicals were supplied by Thermo Fisher Scientific India Pvt. Ltd.

Cell Lines. HEK-293T (human embryonic kidney-293), procured from ATCC, (USA). Dulbecco's Modified Eagle's Medium was used to maintain cells in the proper culture medium and kept at 37°C in an incubator 5% Carbon dioxide (CO_2).

B. Methodology

Preparation of Br-NLCs. The melt-emulsification process, followed by sonication was used to develop the Br-loaded NLCs as per described in our previous reported studies (Singh *et al.,* 2023). Homogeneous lipid phase was produced by melting the solid (capric acid and glyceryl distearate) and liquid (oleic acid) components of the Br (10 mg) lipid phase at 55±5°C. The aqueous phase consisted of distilled water, triethanolamine (20 mg), and phospholipon 90G, which was kept at 55±5°C. The lipid phase was then mixed with the aqueous phase to produce a coarse primary emulsion, and further was followed by sonication (Labsonic® M, Sartorius, Germany) for 10 to 20 minutes (6 cycles with amplitude-80%) to obtain a nanoemulsion. The NLCs dispersion was then quickly congealed over a water bath containing ice and stored at 4°C for further usage (Tripathi *et al.,* 2020).

Surface Decoration of Br-NLCs. The surface decoration of Br-NLCs was completed employing Layer by layer (LBL) approach. Both NLCs and dextrose are negatively charged so a direct coating of dextrose over the NLCs surface is not possible owing to opposite charge repulsion. Thus, for coating an in-between intermediate layer of positively charged polymer is required which can bind to both dextrose and NLCs. For LBL coating Br-NLCs were added into chitosan solution (0.01% w/v) in a dropwise manner (3:1), with constant stirring (Tarsons-Spinot MC 01,

Delhi, India) to acquire Chitosan (CH) layered NLCs (Br-CH-NLCs). The change in surface charge (zeta potential) was then measured confirming the coating as indicated by charge reversal. After charge reversal, the chitosan-coated NLCs were added to dextrose solution (0.01% w/v) in a 3:1 ratio to obtain Br loaded dextrose (DEX) decorated chitosan NLCs (DEX-Br-NLCs), and the charge reversal was confirmed through Zeta potential.

DEX-Br-NLCs Characterization.

1. Particle size, Polydispersity index (PDI), and Zeta potential: To estimate the particle size and PDI of the DEX-Br-NLCs NLCs Zetasizer (Nano plus-3, Yokohama, Japan) was used as per the reported method. All measurements were made in triplicates.

2. Surface Morphology: The transmission electron microscopy was carried out on JEOL 1230 TEM. Briefly, the samples were dispersed in ethanol and positioned on a copper grid and dried at room temperature. The dried grid was loaded on the sample holder and visualised (Sabeti *et al.,* 2014).

3. Entrapment Efficiency and Drug Loading: According to a method previously described EE and drug loading was assessed (Uprit *et al.,* 2013). The centrifugation technique was used to determine drug EE. DEX-Br-NLCs formulation (1 ml) was added to 10 ml of 7.4 pH buffer and DMSO (1:1 mixture) and allowed to stand for 15 minutes. The mixture was then centrifuged for 45 minutes at 25ºC at 8,000 rpm in a micro centrifuged (Sigma Laboratory Centrifuge 4K15). After that, the supernatant's Br concentration was determined by a UV-visible spectrophotometer at 348 nm. Equations were used to compute drug entrapment efficiency and drug loading.

$$\text{Entrapment Efficiency (\%)} = \frac{\text{Amount of drug in NLCs}}{\text{Total amount of drug added}} \times 100$$

$$\text{Drug loading (\%)} = \frac{\text{Amount of drug entrapped in NLCs}}{\text{Total amount of lipid in NLCs}} \times 100$$

4. *In-vitro* Release Study: For release studies of DEX-Br-NLCs, the reported dialysis method was followed (Singh *et al.,* 2022). Dialysis membrane (12–14 kDa, Himedia, India), phosphate buffer saline (PBS) solution (pH 7.4), and gastric buffer (pH 1.2) were used to study the Br release (Singh *et al.,* 2017). The beakers were put together on top of a magnetic stirrer and stirred continuously at a speed of 50rpm and a temperature of 37±2°C. At fixed time points, samples were withdrawn and replaced with an equal quantity of fresh buffer to uphold the sink condition. The Br released at each time point was analyzed using UV spectrophotometry at 348 nm.

Cell line study on HeK293T cells.

1. Cell Viability Assay: The viability assay was accomplished on HEK-293T (human embryonic kidney-293), procured from ATCC, USA. The cell viability was assessed via MTT assay in which 1×10^4 HEK-293T cells/well were seeded into a 96-well plate and cultured in DMEM media maintained at 5% CO_2 and 37°C for 24 h. Br and DEX-Br-NLCs were incubated with the cells at concentrations stretching from 6.25 to 100 µg/ml for 24. After the completion of cell treatment, the MTT dye was used to detect the cell cytotoxicity at 540 nm (Zhang *et al.,* 2021).

2. Cellular uptake study: The protocol reported by Kanoujia *et al.* (2016) was followed while performing cellular uptake of Rhodamine labeled DEX-Br-NLCs. To determine the cell internalization, HEK 293T cells were incubated with Rhodamine labeled DEX-Br-NLCs (100 µg/ml) at 37 °C ± 2 °C with a 5% CO_2 atmosphere in a CO_2 incubator for 24 h. After that, the washing of cells was done using PBS, and further, with 4% paraformaldehyde, fixation was done. After washing, slides were prepared and images were acquired using confocal laser scanning microscopy (LSM900, Zeiss, Munich, Germany), at 546 nm as excitation wavelength and 568 nm as emission wavelength for Rhodamine B (Augustine *et al.,* 2015).

Stability analysis of NLCs. The physicochemical stability study of DEX-Br-NLCs formulation was determined as per International Conference on Harmonization (ICH) guidelines (ICH Q1A(R2) for 6 months. To undertake a storage stability investigation, DEX-Br-NLCs were also kept at 5 ± 3 °C, 25 ± 2 °C/60 ± 5% RH, 40 ± 2 °C/75% ± 5% RH. The DEX-Br-NLCs were assessed for drug loading and particle size. Every experiment was carried out in triplicate (Tripathi *et al.,* 2018).

Statistical analysis. The ANOVA approach was used for the statistical analysis, and the results were given as mean ± standard deviation (SD) (n=3). Levels of significance were determined at $p<0.05$, which explains why Br and DEX-Br-NLCs had significantly different therapeutic efficacies.

RESULT AND DISCUSSIONS

In the current investigation, the charge generated by the CH and DEX was employed for coating on the surface served as confirmation that dual surface modification via LBL method had been carried out. The zeta potential was then assessed at each stage of the creation of surface decorated Br-NLCs were discovered to have a zeta potential of −22.63 ± 0.54 mV. The change in charge of the Br-CH-NLCs, which occurred as a result of chitosan coating, from −22.63±0.54 mV to +21.49±0.54 mV, as shown in (Fig. 1), served as confirmation that the modification had occurred.

At the final step, DEX decoration causes a reversal of charge to the negative from 21.49±0.54 mV to −23.83±0.32 mV due to its cationic nature. Although it is frequently believed that particles must have a zeta potential of less than ±20 mV to be stable, stable NLCs with significantly lower zeta potential has also been observed. In the current work, DEX-Br-NLCs with surface decorations were fabricated and afterward tested for surface morphology (Fig. 1).

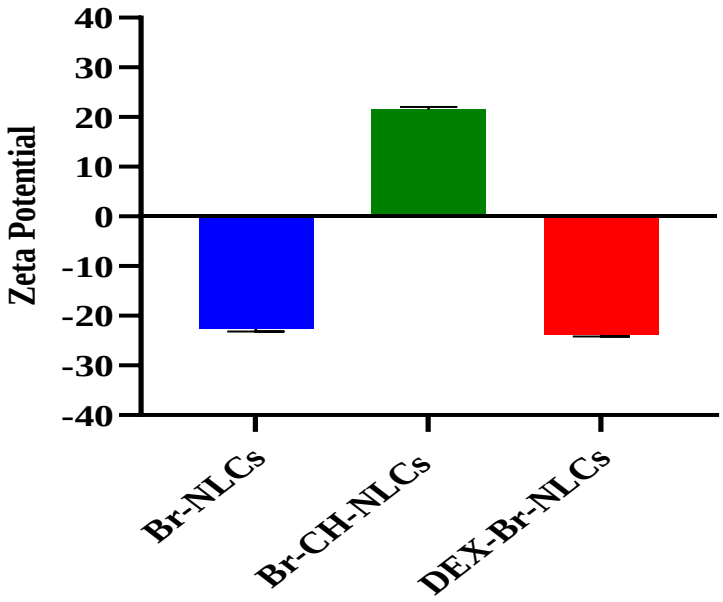
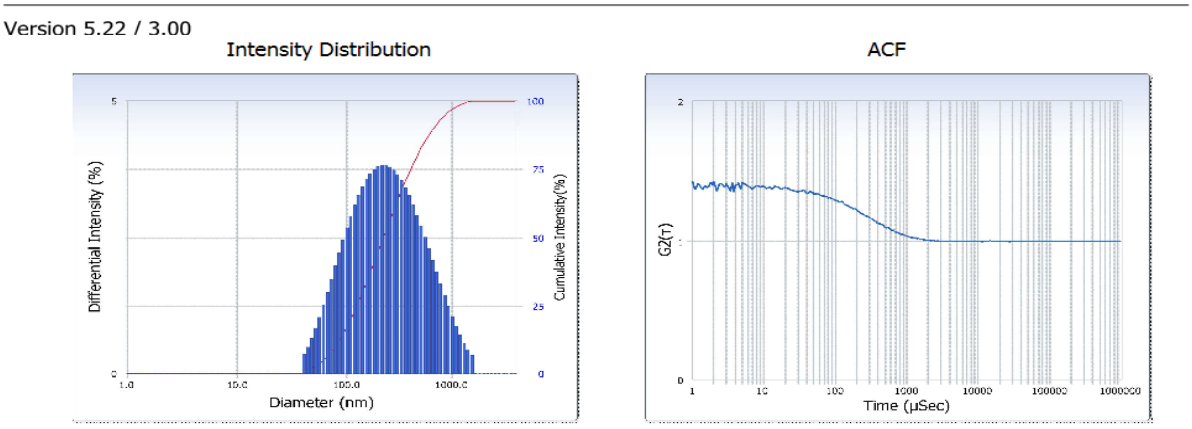


Fig. 1. Zeta potential of Br-NLCs, Br-CH-NLCs, and DEX-Br-NLCs.

A. Particle size, polydispersity index, and zeta potential
The Br NLCs particle size was determined to be 174.1±0.51nm, with an excellent polydispersity index of 0.221**,** demonstrating that the formulations were homogeneous in particle size. The formulations in the current investigation were stable having a zeta potential value of −23.83±0.32 mV whereas in case on coating with DEX slight increase in particle size was found around 200.9±0.53nm with an polydispersity index of 0.281 (Fig. 2). Results of mean diameter analysis showed that nanocarriers were successfully formulated.

B. Transmission electron microscopy analysis (TEM)
Fig. 3 shows TEM (JSM 6100 JEOL, Tokyo, Japan) photomicrographs of DEX-Br-NLCs. The particle size and shape of DEX-Br-NLCs were investigated through TEM. TEM was employed to observe the surface of the NLCs. It exhibited NLCs with smooth, somewhat spherical surfaces.



Distribution Results (Contin)

Peak	Diameter (nm)	Std. Dev.
1	334.1	275.9
2	0.0	0.0
3	0.0	0.0
4	0.0	0.0
5	0.0	0.0
Average	334.1	275.9

Residual : 8.572e-003 (O.K)

Cumulants Results

Diameter	(d)	: 200.9	(nm)
Polydispersity Index	(P.I.)	: 0.281	
Diffusion Const.	(D)	: 2.454e-008	(cm²/sec)
Molecular Weight		: 1.660e+009	
Measurement Condition			
Temperature		: 25.1	(°C)
Diluent Name		: WATER	
Refractive Index		: 1.3328	
Viscosity		: 0.8858	(cP)
Scattering Intensity		: 20689	(cps)
Attenuator 1		: 0.052	(%)

Fig. 2. Particle size, and PDI of DEX-Br-NLCs.

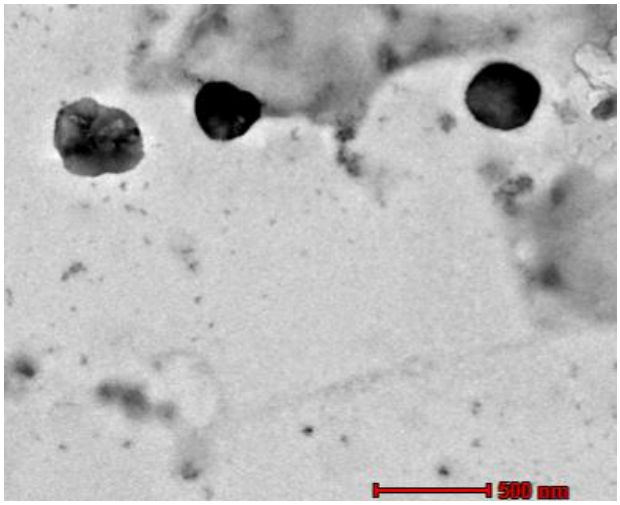


Fig. 3. Showing TEM photomicrographs of DEX-Br-NLCs.

C. Entrapment Efficiency and Drug loading

By assessing the DL potential and EE in Br-NLCs, the formulations were optimized. Br-NLCs, Br-CH-NLCs, and DEX-Br-NLCs were reported to have entrapment and loading efficiencies of 75.46±3.63% and 4.69±0.22%, 72.5±1.02% and 3.2±0.14% and 68.42±1.51% and 3.58±0.14%, respectively. The fabrication stages that result in drug loss during preparation can be responsible for the progressive decline in entrapment efficiency. According to earlier reports, drug entrapment into nanoparticles not only enhanced the drug's therapeutic impact but also prolonged the drug's effectiveness since nanoparticles have a longer resistance period in the blood circulatory system than free drugs.

D. In vitro Drug release

The DEX-Br-NLCs formulation displayed prolonged drug release when the cumulative release was contrasted to the pure drug. The initial fast release of the pure drug was followed by a maximal release of up to 65-70% in 4h. But the DEX-Br-NLCs formulation demonstrated about 45–50% drug release in 4h and 80.32±6.65% drug release over 24 hours. The DEX-Br-NLCs formulation released 50% of the drug in 12h, with a maximum release of 60.24 ± 4.43% at the end of 24h, according to the dissolution of the NLC in gastrointestinal pH (acidic buffer pH 1.2) (Fig. 4). Following the 12-hour mark, the drug release remained nearly steady until the study's end. Additionally, in comparison to the release profile at pH 7.4, no discernible difference in the drug release profile was seen. At pH 1.2, there was no burst release, which is another sign that the DEX-Br-NLCs formulation is stable at the pH of the stomach (Sahibzada *et al.,* 2018).

E. Cell viability

The viability of HEK 293T cells was assessed through the MTT test after being exposed to Br and Br NLCs for the incubation times of 24 hours. After a 24h incubation period, the MTT assay showed that DEX-Br-NLCs had cell viability that was 97.1±1.65% higher than Br (91.1 ± 1.66%) at the maximum concentration of 100µg/ml (Fig. 5). On the other hand, the Br NLCs (6.25 µg/ml-100 µg/ml) maintained good cell viability (about 97%) at contact durations of 24 h and demonstrated nontoxicity of NLCs at elevated concentrations (White *et al.,* 2022).

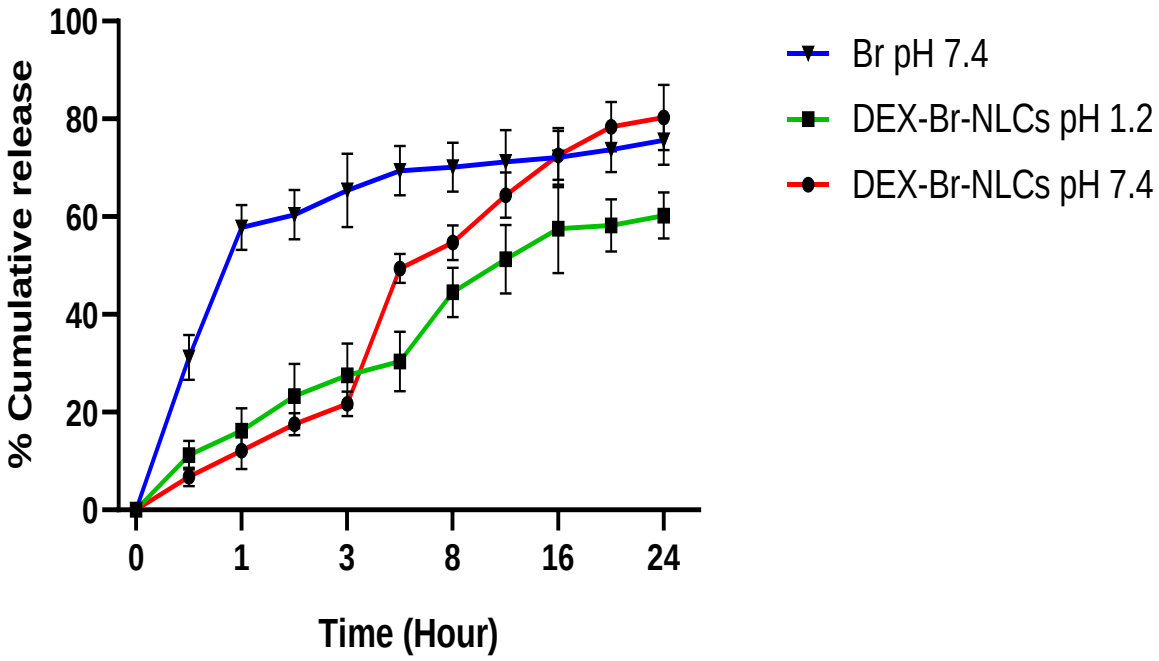


Fig. 4. Br and DEX-Br-NLCs release profiles in vitro at pH 1.2 and 7.4. Data are represented as the (mean±SD, n=3).

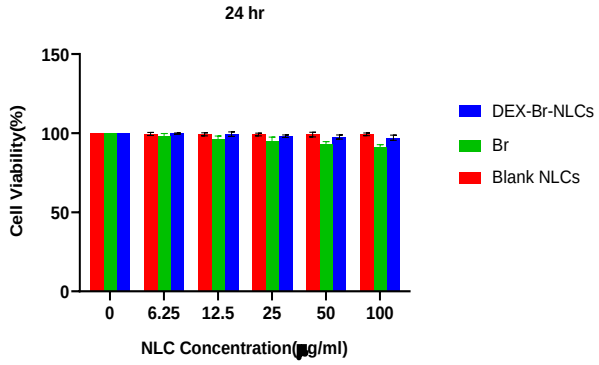


Fig. 5. Cell viability of DEX-Br-NLCs, Br, and Blank NLC, and determined by MTT assay. Data are expressed as mean ± SD of four independent experiments.

F. Cellular uptake of Br-loaded NLCs

Cellular uptake of Rhodamine labeled DEX-Br-NLCs by HEK 293T cells were determined through confocal laser scanning microscopy (Fig. 6) (Deng *et al.*, 2020). Confocal microscopy pictures amply demonstrated the NLCs' qualitative cellular uptake. The red channel showed that the cytoplasm of the cell was where Br NLCs were primarily concentrated. The discovery that small molecules primarily diffuse into cells but endocytosis is the mechanism by which nanocarriers are absorbed may account for the internalization of NLCs (Paudel *et al.*, 2022).

G. Stability studies

Dex-Br-NLCs formulation was found to be stable as per the specified guidelines and negligible changes were observed in drug loading and mean diameter (Table 1). No significant changes were observed in mean diameter and drug loading when formulations were stored for 6 months (Santamaría-Aguirre *et al.*, 2018).

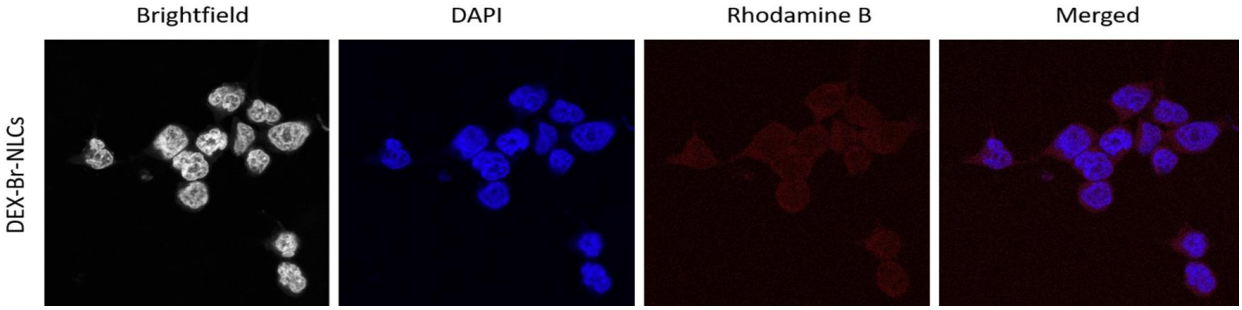


Fig. 6. Rhodamine B labeled DEX-Br-NLCs showing Intracellular uptake in HEK 293T cell lines with the help of confocal microscopy at 40x magnification.

Table 1: Stability studies of DEX-Br-NLCs.

Sampling intervals (months)	Particle Size (nm)			% Drug loading		
	5 ± 3 °C	25 ± 2 °C	40 °C ±2°C	5 ± 3 °C	25°C±2°C	40 °C ±2°C
0	201.4 ± 2.13	201.4 ± 2.13	201.4 ± 2.13	3.52 ± 1.35	3.52 ± 1.35	3.52 ± 1.35
2	204.2 ± 3.47	210.4 ± 3.64	214.5 ± 3.27	3.47 ± 0.59	3.48 ± 0.67	3.25 ± 0.85
4	208.7 ± 3.24	213.5 ± 3.11	217.1 ± 4.02	4.45 ± 1.13	3.44 ± 0.48	3.07 ± 0.76
6	211.5 ± 3.16	215.4 ± 2.17	222.2 ± 3.25	4.05 ± 1.34	3.03 ± 0.37	2.89 ± 1.07

CONCLUSIONS

In summary, DEX-Br-NLCs were prepared successfully. Results indicate that the entrapment of Br into NLCs showed great potential to deliver the drug efficiently to the predetermined desired site witnessing shielding effect on the encased drug during transit against the wide pH variability throughout GIT, HEK293T cells showed no significant toxicity. The in vitro release studies displayed that the formulated lipid matrix delivered a controlled release drug and the stability studies demonstrated that NLCs were stable when stored for a long period.

FUTURE SCOPE

The prepared nanoformulations can be explored for preclinical and clinical investigations and can be scaled up for market authorization and finally human use.

Conflict of Interest. The authors report no conflicts of interest.

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