ABSTRACT

Breast cancer remains a significant global health burden, with increasing incidence and mortality rates necessitating the development of novel therapeutic strategies. Conventional chemotherapy, despite its effectiveness, is limited by poor bioavailability, systemic toxicity, and the emergence of resistance mechanisms. Paclitaxel (PAC) and Cyclophosphamide (CYC) are widely used chemotherapeutic agents in breast cancer treatment; however, their clinical utility is hindered by rapid clearance, poor solubility, and adverse systemic effects. The **aim of present research** focused on the development of two separate drug delivery systems i.e. nanostructured lipid carriers (NLCs) and microemulsion (MEs) for the simultaneous delivery of PAC and CYC for the treatment of Breast Cancer.The **present research hypothesised** that Nanostructured Lipid Carriers (NLCs) and Microemulsion (MEs) containing co-loaded PAC and CYC will improve drug solubility, stability, provide targeted delivery, and enhance therapeutic efficacy while overcoming drug resistance mechanisms in breast cancer treatment.

A Quality by Design (QbD) integrated with OFAT (One Factor At a Time) approach was employed to systematically optimize the formulation and process parameters of PAC-CYC NLCs and MEs. As a part of QbD approach, formulation development contained elements delineated in ICH Q8 (R2): i.e. [A] Quality Target Product Profile (QTPP) [B] Critical Quality Attributes (CQAs) of the drug product [C] Qualitative risk assessment using Ishikawa diagram Preformulation studies were conducted to select suitable lipids and oils, surfactants, and co-surfactants, ensuring maximum drug loading and stability.

NLCs were formulated and developed in lyophilized form. Redispersibility of less than 90 seconds with Z-average of 53.9 ± 2.0nm, PDI of 0.125 ± 0.018 and zeta potential of -5.3 ± 2.7 mV was achieved for developed PAC-CYC NLCs. NLCs demonstrated high entrapment efficiency of > 95% for both the drugs with assay in the range of 90.0 to 110.0 % even throughout the stability. The water content of lyophilized NLCs was observed to be 0.5± 0.1 %. The FTIR analysis of developed NLCs exhibited slight shift in the characteristic peaks of both the drugs which confirmed the encapsulation of both the drugs as such shifts are typical when drugs are encapsulated in lipid-based carriers, as lipid matrix can influence drug's vibrational properties.

DSC thermogram of NLCs did not show the melting peak of Paclitaxel and Cyclophosphamide suggesting that Paclitaxel and Cyclophosphamide were present in the NLCs either in amorphous form or molecularly dispersed. This encapsulation leads to improved solubility and stability, which is a key advantage of using NLCs for drug delivery. XRD is a crucial analytical technique for characterizing lyophilized NLCs due to its ability to provide detailed insights into the crystallinity and structural properties of these systems. The amorphous behaviour was observed for PAC-CYC NLCs in XRD spectra, as opposed to the crystalline nature of pure Paclitaxel and Cyclophosphamide. It can be attributed to the molecular dispersion or amorphous form of these drugs within the nanostructured lipid carriers (NLCs). This transformation is often a result of encapsulation process, which alters molecular arrangement and prevents formation of a crystalline lattice.Transmission Electron Microscopy (TEM) confirmed successful nanoscale particle formation whereas in-vitro drug release studies confirmed that Paclitaxel and Cyclophosphamide loaded in NLCs required 24 hours for cumulative drug release of 96.07 ± 0.70 % and 98.37 ± 0.35 % respectively which ultimately bypassed the limitations of conventional therapies-namely rapid clearance and systemic toxicity.The stability studies of developed NLCs demonstrated sterility and sustained integrity over 12 months at 2 to 8°C.

PAC-CYC loaded microemulsions demonstrated excellent stability upon dilution at ratios of 1:10, 1:100, and 1:250, exhibiting no indications of phase separation, precipitation, or breaking proving that the emulsion was classified as oil-in-water. The viscosity of optimized microemulsion was reported as 1.09 ± 0.06 cps. The system exhibits characteristics akin to a homogeneous solution, indicating that the viscosity remains low and comparable to that of water. The Z-average of 62.8 ± 1.0nm with PDI of 0.142 ± 0.031 indicated that size distribution is narrow whereas the zeta-potential of microemulsion was reported as -2.6 ± 0.2 mV.TEM revealed that optimized microemulsion were discrete and spherical oil globules dispersed in continuous phase of microemulsion. The entrapment efficiency of Paclitaxel was obtained as 99.7±0.2 % whereas for Cyclophosphamide it was obtained as 100.6±0.8 %. Assay of Paclitaxel in microemulsion was determined as 99.0 ± 0.4 % and assay of Cyclophosphamide in microemulsion was reported as 100.8 ± 0.3 %. Cumulative drug release of 98.27 ± 0.75 % and 96.87 ± 0.50 % was observed for both the Paclitaxel and Cyclophosphamide marketed formulations within 3 hours respectively whereas, Paclitaxel and Cyclophosphamide loaded in microemulsion required 6hours for cumulative drug release of 87.89 ± 2.49 % and 96.10 ± 0.75 %respectively, showcasing that microemulsion had slower drug release profile. The stability

studies of developed microemulsion demonstrated sterility and sustained integrity over 12 months at 2 to 8°C.

Conventional chemotherapeutics often exhibit a short systemic half-life due to rapid renal/hepatic clearance and non-specific biodistribution. The NLCs and MEs demonstrated controlled and sustained drug release profiles, as described in the in-vitro release studies. These nanocarriers increased circulation time through their optimized particle size (typically <200 nm) and surface properties, allowing enhanced permeability and retention (EPR) effect at tumor sites. Lipid-based nanocarriers inherently avoid immediate uptake by the reticuloendothelial system (RES), thereby prolonging plasma residence time. Issue of poor solubility, a problem with conventional formulations was resolved by the use of lipid based formulation systems. The NLCs encapsulated the dual drugs within their solid & liquid lipid matrix, significantly enhancing its aqueous dispersability.Microemulsionssolubilized both drugs effectively through a thermodynamically stable oil-surfactant-water interface, improving solubility and bioavailability**.**Conventional formulations cause off-target toxicity due to non-specific distribution and high peak plasma concentrations. Targeted delivery was achieved by size-controlled nanocarriers that preferentially accumulate in tumor tissues via passive targeting (EPR effect). In-vitro cytotoxicity studies on cancer cell lines showed enhanced tumor-killing efficiency of the dual-drug-loaded nanocarriers at lower doses compared to free drugs, indicating improved therapeutic index.

Cytotoxicity studies using MCF-7 breast cancer cell lines indicated that PAC-CYC NLCs and MEs significantly reduced IC_{50} values relative to free PAC and free CYC. Cellular uptake studies demonstrated increased intracellular drug accumulation, while apoptosis assays confirmed higher rates of cell death and enhanced G2/M phase cell cycle arrest in cells treated with nanocarrier formulations, suggesting reduced likelihood of resistance development.

In-vivo efficacy studies using a xenograft breast cancer model in female Sprague Dawley rats validated the therapeutic superiority of PAC-CYC NLCs and MEs. Tumor regression studies demonstrated significant inhibition of tumor growth in nanocarrier-treated groups compared to conventional chemotherapy. Histopathological analysis revealed a marked reduction in

tumor cell infiltration, lower inflammatory response, and preservation of normal tissue architecture in the NLC and ME-treated groups. These findings underscore the potential of nanocarrier-based drug delivery systems to enhance the therapeutic index of PAC and CYC while minimizing systemic toxicity.

The simultaneous incorporation of Paclitaxel and Cyclophosphamide within individual nanocarriers—Nanostructured Lipid Carriers (NLCs) and Microemulsions (MEs)—represents a significant formulation challenge that is elegantly solved in this study. This dual-drug co-loading ensures synergistic therapeutic action, with both agents targeting different stages of the cell cycle (S-phase and G2/M phase), thereby maximizing antitumor efficacy while minimizing resistance. The successful co-delivery platform eliminates the need for toxic solubilizers (e.g., Cremophor EL) and supports dose minimization.

The physicochemical characteristics and therapeutic effectiveness of dual-drug-loaded formulations were evaluated to ascertain the optimal delivery method. The efficacy and dosage volume of a delivery system are significantly influenced by its loading capacity. Microemulsions demonstrated higher drug loading capacities than NLCs. The superior loading in microemulsions is due to their isotropic structure, which provides extensive interfacial areas and surfactant-rich domains. Particle size critically affects biodistribution, cellular uptake, and clearance of nanocarriers. NLCs had a smaller Z-average size than microemulsions. This minor size advantage positions NLCs within the ideal range for enhanced tumor penetration via the EPR effect. Additionally, NLCs exhibited a lower PDI, reflecting a more uniform size distribution and enhanced colloidal stability. NLCs possessed a greater negative surface charge compared to microemulsions, though both values are relatively low and similar. High entrapment efficiency is essential for optimizing therapeutic load while minimizing toxicity.

Both formulations displayed excellent entrapment efficiencies for PAC and CYC, suggesting suitability for drug encapsulation. Assay results corroborated these findings, confirming consistency in drug loading. Drug release kinetics are vital for therapeutic effectiveness and dosing schedules. NLCs exhibited a more sustained release profile compared to the rapid release observed in microemulsions. The sustained release from NLCs is attributed to the solid lipid matrix, contrasting with the quicker release from the liquid nature of

microemulsions. This sustained release is particularly beneficial for chemotherapeutics, enhancing compliance by reducing dosing frequency. NLCs' prolonged release kinetics support sustained therapeutic levels, while microemulsions are advantageous for rapid action onset. Cell viability assessments indicated similar cytotoxicities for both the formulations, affirming effective drug delivery and bioavailability. The pivotal distinction was observed in the in-vivo antitumor efficacy; microemulsions led to complete tumor cell absence, whereas NLCs showed minimal detectable tumor presence. This suggests a superior therapeutic effect of microemulsions potentially due to their rapid drug release, which is advantageous for aggressive tumors. However, the choice between sustained action and immediate effect presents a trade-off: NLCs may maintain drug efficacy with fewer doses, while microemulsions may necessitate frequent administration to sustain therapeutic benefits.

The comparative evaluation of Paclitaxel and Cyclophosphamide co-loaded Nanostructured Lipid Carriers (NLCs) and Microemulsions (MEs) revealed that both platforms are highly competent for dual-drug delivery in cancer therapy.From a physicochemical perspective, NLCs demonstrated smaller particle size, and sustained drug release, making them suitable for prolonged systemic circulation and controlled release applications. These characteristics are highly desirable for chronic chemotherapy regimens requiring reduced dosing frequency and enhanced tumor targeting. On the other hand, Microemulsions offered a slightly superior immediate therapeutic effect, as evidenced by complete tumor clearance in-vivo, likely due to their faster release kinetics. This formulation may thus be better suited for acute tumor burden reduction where rapid drug action is essential.Therefore, the selection between NLCs and microemulsions should be guided by the therapeutic goal—NLCs for sustained delivery and patient compliance, and microemulsions for rapid onset of action. Future studies may explore a hybrid approach combining either systems or sequential dosing to leverage the advantages of each.

The nanocarriers are developed using scalable techniques demonstrating the industrial viability of these formulations. Emphasis on physicochemical characterization, storage stability, and aligns with regulatory and manufacturing standards, facilitating smoother bench-to-clinic translation. The work is grounded in a mechanistic rationale that addresses multidrug resistance (MDR) and tumor heterogeneity by co-delivering agents with complementary mechanisms of action, enhancing intracellular drug concentration and

targeting multiple signaling pathways simultaneously to reduce mutation-driven escape. The research integrates extensive characterization (particle size, zeta potential, PDI, drug loading, release kinetics, and stability) with biological validation (in-vivo efficacy studies), including in-vitro cytotoxicity studies, which together build a compelling translational case. This bridging of pharmaceutical technology with preclinical oncology exemplifies an interdisciplinary approach.

In conclusion, the developed NLCs and MEs present a viable nanocarrier platform for the co-delivery of PAC and CYC, addressing key challenges in breast cancer treatment. The study successfully demonstrated improved drug solubility, prolonged in-vitro release kinetics, increased tumor targeting, and enhanced therapeutic efficacy, paving the way for potential clinical translation of these advanced drug delivery systems.