Journal of Young Pharmacists, 2026; 18(2):369-376.
https://www.jyoungpharm.org

Original Article

Synthesis and *in vitro* Evaluation of Folic Acid-Functionalized Liquid Crystalline Nanoparticles for Enhanced Cytotoxicity in HCT-116 and HT-29 Colorectal Cancer Cells

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ABSTRACT

Background: Colorectal Cancer (CRC) therapy is limited by poor tumor selectivity and systemic toxicity associated with 5-Fluorouracil (5-FU). Liquid Crystalline Nanoparticles (LCNs) offer sustained drug release and improved intracellular delivery, while Folate Receptor (FR)-mediated targeting may further enhance therapeutic specificity. This study aimed to develop and evaluate Folic Acid-Functionalized LCNs (FA-LCNs) loaded with 5-FU, Melatonin (MEL) for improved cytotoxic efficacy against CRC cells. **Materials and Methods:** Folic acid was conjugated to Poloxamer 407 (FA-P407) using Carbonyldiimidazole (CDI) mediated coupling and characterized by FT-IR and DSC to confirm amide bond formation. Optimized LCNs and FA-LCNs co-loaded with 5-FU and MEL were prepared using a Design of Experiments (DoE) approach. Physicochemical properties, including particle size, zeta potential, and Polydispersity Index (PDI), were evaluated. *In vitro* cytotoxicity was assessed in HCT-116 and HT-29 colorectal cancer cells using the MTT assay. **Results:** Successful FA conjugation was confirmed by characteristic amide bond formation and altered thermal behavior. LCNs exhibited a particle size of 187.8 nm, which slightly increased to 201.8 nm after folate functionalization, with enhanced surface charge (-48.8 mV) indicating improved stability. FA-LCNs demonstrated significantly enhanced cytotoxicity, reducing IC_{50} values by approximately 2 to 3 fold compared to free 5-FU, MEL particularly in chemo resistant HT-29 cells. **Conclusion:** FA-LCNs significantly enhance the anticancer efficacy of 5-FU, MEL in HCT-116, HT-29 colorectal cancer cells. FA-mediated targeting improves cytotoxic potency against chemo resistant HT-29 cells, suggesting potential utility in overcoming partial drug resistance. However further extensive study warranted to support these findings.

Keywords: 5-Fluorouracil, Colorectal Cancer, Folic Acid Conjugation, Liquid Crystalline Nanoparticles.

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Received: 16-12-2025;
Revised: 06-02-2026;
Accepted: 27-03-2026.

INTRODUCTION

Colorectal Cancer (CRC) remains one of the most prevalent malignancies worldwide and a leading cause of cancer-related mortality (Rawla *et al*., 2019). Although advances in surgical resection and combination chemotherapy have improved survival outcomes, therapeutic efficacy is often limited by systemic toxicity, poor tumor selectivity, inadequate intracellular drug retention, and the development of chemoresistance (Al-Jaber *et al*., 2025). 5-Fluorouracil (5-FU) continues to serve as a first-line chemotherapeutic agent in CRC management; however, its short biological half-life, rapid metabolic degradation, and non-specific biodistribution restrict its therapeutic index and frequently necessitate high systemic dosing (Mazzuca *et al*., 2016). These limitations underscore the need for advanced drug delivery systems capable of enhancing tumor-targeted accumulation while minimizing off-target toxicity. Nanotechnology-based delivery platforms have emerged as promising strategies to improve pharmacokinetics, tumor localization, and controlled drug release. Among these, lipid-based nanocarriers are particularly attractive due to their biocompatibility, biodegradability, and strong interaction with biological membranes (Yang and Merlin, 2020). Liquid Crystalline Nanoparticles (LCNs), including cubosomes and hexosomes, represent a distinct class of lipid nanocarriers characterized by highly ordered cubic or hexagonal internal nanostructures. Their bicontinuous architecture provides extensive internal surface area and interconnected aqueous channels, enabling efficient encapsulation of both hydrophilic

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DOI: 10.5530/jyp.20260057

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and lipophilic agents with sustained release behavior (Zhang *et al.*, 2024). The thermodynamically stable liquid crystalline phase further enhances structural integrity and minimizes premature drug leakage, supporting prolonged intracellular exposure.

While passive tumor accumulation through nanosystem localization alone may not ensure optimal specificity in heterogeneous solid tumors (Khan *et al.*, 2026). Active targeting strategies therefore offer additional precision by functionalizing nanocarrier surfaces with ligands that recognize tumor-associated receptors. The Folate Receptor (FR), overexpressed in several epithelial malignancies including colorectal adenocarcinoma, represents a well-established target for receptor-mediated endocytosis (Zhang *et al.*, 2025). Surface conjugation of Folic Acid (FA) enhances selective cellular uptake and intracellular drug accumulation, thereby improving cytotoxic efficacy in FR-positive cancer cells.

Poloxamer 407 (P407), a triblock copolymer composed of Poly(Ethylene Oxide)-Poly(propylene Oxide)-Poly(Ethylene Oxide) (PEO-PPO-PEO), is widely employed in nanocarrier systems due to its amphiphilicity, steric stabilization capability, and biocompatibility (Zarrintaj *et al.*, 2020). FA-P407 conjugates have been synthesized using carbonyldiimidazole mediated coupling approaches and characterized using spectroscopic techniques such as FT-IR, DSC, demonstrating enhanced cellular uptake compared with non-functionalized systems. Despite these advancements, many studies primarily focus on formulation optimization, with comparatively limited in-depth molecular characterization of ligand-stabilizer conjugation. Moreover, systematic comparative evaluation between optimized non-targeted systems and ligand-functionalized counterparts under identical experimental conditions remains insufficient. Consequently, the specific contribution of ligand conjugation to improved biological performance is not always clearly delineated.

Therefore, the present study aims to synthesize and structurally characterize a folic acid-conjugated Poloxamer 407 (FA-P407) stabilizer using spectroscopic and thermal analytical techniques to confirm successful conjugation. The conjugated system is subsequently incorporated into liquid crystalline nanoparticles and comparatively evaluated against the optimized non-targeted formulation to determine the impact of folate functionalization on cytotoxic response in folate receptor-expressing colorectal cancer cells.

MATERIALS AND METHODS

Folic acid, Poloxamer P407, and 1,1′-Carbonyldiimidazole (CDI) was obtained from Sigma Aldrich. Dimethyl Sulphoxide (DMSO) was obtained Thermo Fisher Scientific India Pvt. Ltd., 5-fluorouracil (5-FU) and Melatonin was obtained from Tokyo Chemical Industry (TCI) Pvt. Ltd., Glycerol Monooleate (GMO) was a kind gift from Mohini Organic's Private Limited, Mumbai, India. Cell lines: HT-29 and HCT-116 (Human colorectal adenocarcinoma cells) obtained from National Centre for Cell Science (NCCS), Pune, India. And all the other chemicals and reagents used were of analytical grade.

Synthesis of Folic Acid Conjugated Poloxamer 407

Folic acid-Poloxamer 407 (FA-P407) conjugate was synthesized in a manner that was formerly documented (Hou *et al.*, 2020). 10 mL of Dimethyl Sulfoxide (DMSO) were mixed with 175.16 mg of Folic Acid (FA) and stirred until the FA was completely dissolved. The FA solution was mixed with 70.64 mg of Carbonyldiimidazole (CDI) and magnetically stirred for a whole day in the dark to activate the amino terminus of folic acid. 1222 mg of Poloxamer 407 (P407) was added to the reaction mixture and agitated in the dark for a further 24 hr in order to couple folic acid with poloxamer 407. Following this, the reaction mixture was dialyzed against double-distilled water for three days using a dialysis bag. Using lyophilization, the folic acid-Poloxamer 407 (FA-P407) conjugate was recovered**.**

Characterization of FA-P407 by Fourier Transform Infrared Spectroscopy (FT-IR)

A Fourier Transform Infrared Spectroscopy (FT-IR) instrument was used to verify that FA was conjugated to the stabilizer (P407). The Shimadzu FTIR-8400S instrument was used to record the spectra. After the ingredients were combined with potassium bromide and formed into pellets, the equipment was used to record the spectra of FA, P407, and FA-P407 conjugate.

Differential Scanning Calorimetry (DSC)

Using a Mettler-Toledo DSC instrument (Germany), a differential scanning calorimetry (DSC) study was used to characterize the conjugation of folic acid to the stabilizer by analyzing FA, P407, and FA-P407 separately. The samples were then placed inside aluminum pans with pin-hole lids. Dry nitrogen gas was continuously pumped through the DSC chamber to guarantee an inert atmosphere.

Formulation of LCNs and FA-LCNs loaded with 5-FU and MEL

Liquid Crystalline Nanoparticles (LCNs) were prepared using a previously optimized formulation obtained through a Design of Experiments (DoE) approach with DesignExpert software (Stat-Ease, USA) version 13 employing a Box-Behnken design. The optimized formulation parameters, including lipid-to-stabilizer ratio and processing conditions, were selected based on the desired particle size, polydispersity index, and entrapment efficiency. Briefly, Glyceryl Monooleate (GMO) was used as the lipid phase, and Poloxamer 407 (P407) served as the stabilizer. GMO, P-407, MEL were initially melted at 70°C and 5FU was dissolved in aqueous phase separately and maintained at 70°C. The aqueous phase was added gradually in to molten lipid phase under continuous stirring to form a cubic gel. The dispersion

was then subjected to high-speed homogenization (top-down approach) at optimized speed and duration to obtain nanosized liquid crystalline particles.

For the preparation of folic acid-Functionalized Liquid Crystalline Nanoparticles (FA-LCNs), the same optimized formulation and processing conditions were employed. The previously synthesized folic acid-Poloxamer 407 conjugate (FA-P407) was used as the stabilizing agent in place of unmodified P407, maintaining equivalent stabilizer concentration to preserve formulation consistency. The lipid dispersion and high-speed homogenization steps were performed under identical conditions to ensure comparability between non-targeted LCNs and FA-LCNs. The resulting nanoparticle dispersions were allowed to equilibrate at room temperature prior to further characterization and *in vitro* evaluation.

In vitro cytotoxicity assay

DMEM-High Glucose media, 10% FBS, penicillin, and streptomycin at 1X final concentration from a 100X stock were added to the cells, which were kept in a CO_2 incubator setting with 5% CO_2 and 95% humidity. The cells were trypsinized with trypsin-EDTA once they had grown to a confluent state, and the cells were then seeded into sterile 96-well plates to be used in experiments. The cytotoxic potential of the prepared formulations against HCT-116 and HT-29 colorectal cancer cell lines was evaluated using the MTT assay. 5-FU, LCNs, and FA-LCNs were tested in µM/mL and MEL tested in mM/mL concentration. After performing the assay, the cells were visualized using an inverted phase contrast microscope with a magnification of 10x.

RESULTS

Characterization of FA-P407 by Fourier Transform Infrared Spectroscopy (FT-IR)

The folic acid spectra (Figure 1a), poloxamer 407 (Figure 1b), and FA-P407 (Figure 1c) illustrated in Figure 1. The amide bond's creation as evidence that the conjugation was successful.

Differential Scanning Calorimetry (DSC)

Differential Scanning Calorimetry (DSC) of P407 (Figure 2a), folic acid (Figure 2b) and FA-P407 (Figure 2c) illustrated in Figure 2. strong peak observed in the individual folic acid which was disappeared in FA-P407 (Figure 2c) confirms successful conjugation.

Characterization of Prepared LCNs and FA-LCNs

Characterization of the prepared LCNs and FA-LCNs were given in Table 1. The optimized LCNs exhibited a mean particle size of 187.8 nm, which increased slightly to 201.8 nm following folic acid conjugation in FA-LCNs. The zeta potential of LCNs was measured at -35.9 mV, upon folate conjugation, the zeta potential shifted to -48.8 mV, reflecting an increase in negative surface charge. Polydispersity Index (PDI) values were 0.264 for LCNs and 0.284 for FA-LCNs, indicating a relatively narrow size distribution for both systems.

In vitro Cytotoxicity Assessment in HCT-116, HT-29 Colorectal Cancer Cells

The cytotoxicity of free 5-FU, MEL, LCNs, and FA-LCNs was systematically evaluated in HCT-116 (Figure3(A)) and HT-29 (Figure3(B)) colorectal cancer cell lines at 72 hr. 5-FU, LCNs, and FA-LCNs were tested in µM/mL and MEL tested in mM/mL concentration. The representative bright-field microscopic images of cytotoxicity of LCNs (Figure 4(A)), FA-LCNs (Figure 4(B)) against HCT-116 and cytotoxicity of LCNs (Figure 4(C)), FA-LCNs (Figure 4(D)) against HT-29 represented in Figure 4. Note that all treatment groups demonstrated a clear concentration-dependent decline in cell viability, with a more pronounced effect at 72 hr, indicating sustained pharmacodynamic activity consistent with sustained intracellular drug release.

DISCUSSION

Characterization of FA-P407 by FT-IR spectra reveals the folic acid spectra (Figure 1a) peaks at 1484 cm^{-1} corresponds to phenyl ring of folic acid (Ghalehkhondabi *et al.*, 2021). Poloxamer 407 characteristic peak (Figure 1b) was found at 1105 cm^{-1} originating due to the -C-O-C stretch (Lukáčová Bujňáková *et al.*, 2020). It was found that FA-P407 contains the amide group (-CO-NH-) as the N-H stretch was discovered at about 3396 cm^{-1} and the C=O absorption peak was observed at 1510 cm^{-1} and 1692 cm^{-1} as illustrated in the Figure 1c (Pawar *et al.*, 2018; D. Zhang *et al.*, 2015) which confirms the conjugation was successful. DSC curve revealed that there is a strong endothermic peak at about 60°C (Figure 2a), which is the characteristic melting point of P407. At 150°C, a peak was seen, as the melting of the folic acid crystalline component (Figure 2b). It appears that the generated conjugated product is a single compound and not a mixture of both folic acid and poloxamer because, in the case of conjugated poloxamer, a peak associated with poloxamer 407 was found, but the strong peak observed in the individual folic acid disappeared (Figure 2c). Folic acid has successfully conjugated to Poloxamer

Table 1: Particle size, Zeta potential, PDI analysis of LCNs, FA-LCNs.

Particle size (nm)		Zeta potential (mV)		PDI	
LCNs	FA-LCNs	LCNs	FA-LCNs	LCNs	FA-LCNs
187.8	201.8	-35.9	-48.8	0.264	0.284

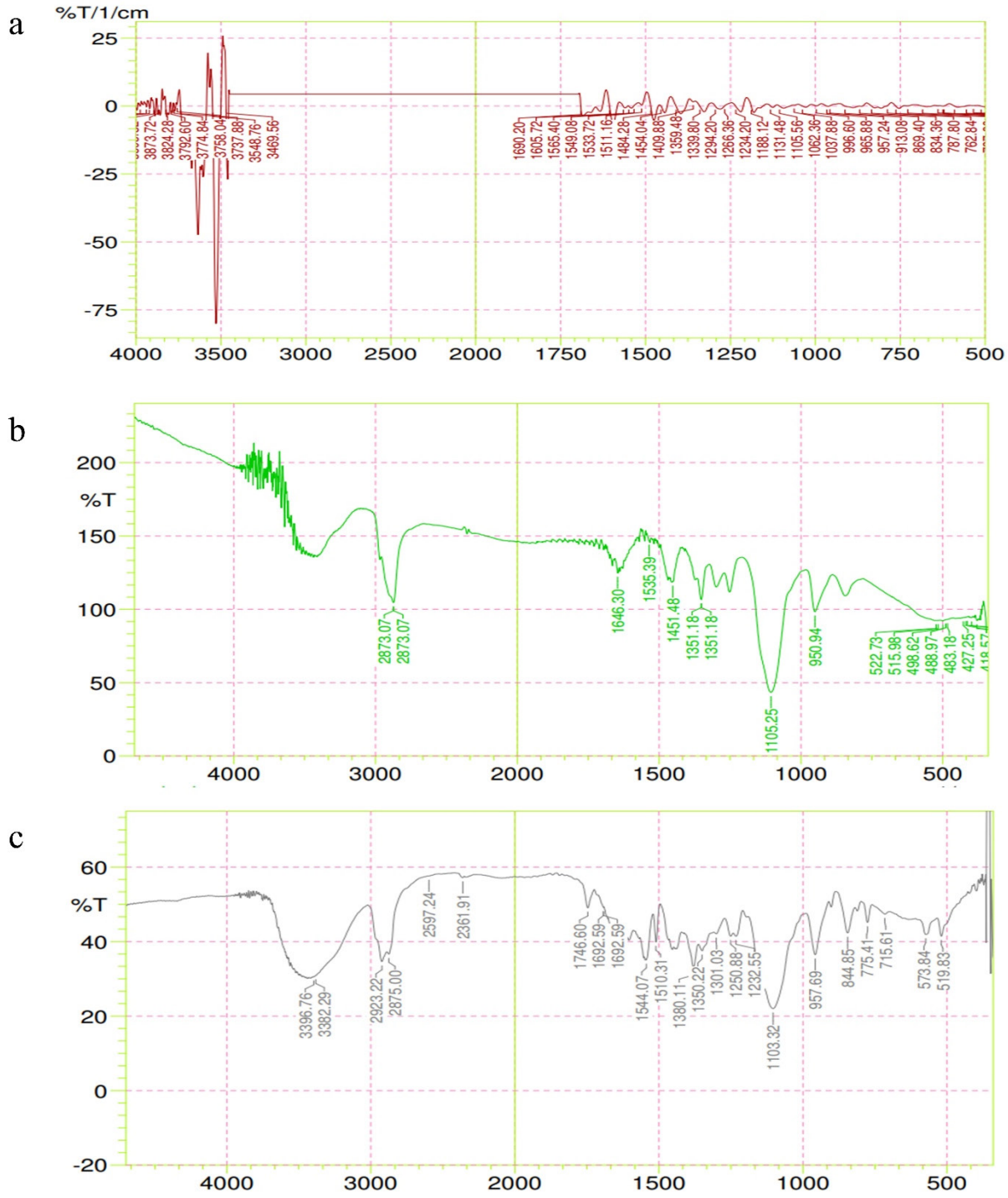

Figure 1: Infra-red spectral profiles of (a) P407 (b) FA and (c) FA-P407.

407, as evidenced by the alterations in the conjugate's DSC curves as compared to its individual component parts as depicted in the Figure 2 (Zhang *et al.*, 2015). Characterization of prepared LCNs and FA-LCNs exhibited there is modest increase (14 nm) in particle diameter of FA-LCNs compared to LCNs confirms successful surface functionalization of the nanoparticles without causing substantial aggregation or destabilization. The size of both formulations remained within the nanoscale range (< 250 nm), which is favorable for enhanced tumor accumulation via the Enhanced Permeability and Retention (EPR) effect and efficient cellular internalization. Upon folate conjugation, the zeta potential shifted to -48.8 mV, reflecting an increase in negative surface charge. This shift likely arises from the presence of ionizable carboxyl groups associated with folic acid on the nanoparticle surface, providing indirect evidence of successful ligand attachment. The higher magnitude of negative zeta

potential in FA-LCNs suggests improved colloidal stability and reduced likelihood of particle aggregation. Overall, the physicochemical characterization demonstrates that folate functionalization resulted in a controlled increase in particle size and surface charge without compromising nanoscale dimensions or dispersion uniformity. These findings confirm the successful development of a stable, ligand-functionalized nanocarrier suitable for targeted delivery applications.

In vitro cytotoxicity against HCT-116 at 72 hr represented in Figure 3(A) displayed 5-FU induced moderate growth inhibition yielding an estimated IC_{50} of 8.36 µM at 72 hr. Melatonin exhibited comparatively limited cytotoxicity within the tested concentration range supporting its adjunctive rather than primary cytotoxic role in colorectal cancer therapy (Reiter *et al.*, 2017). Encapsulation of the drug within LCNs shown better antiproliferative activity, reducing the IC_{50} to approximately 7.59 µM. This nearly 1.1-fold improvement suggests that the

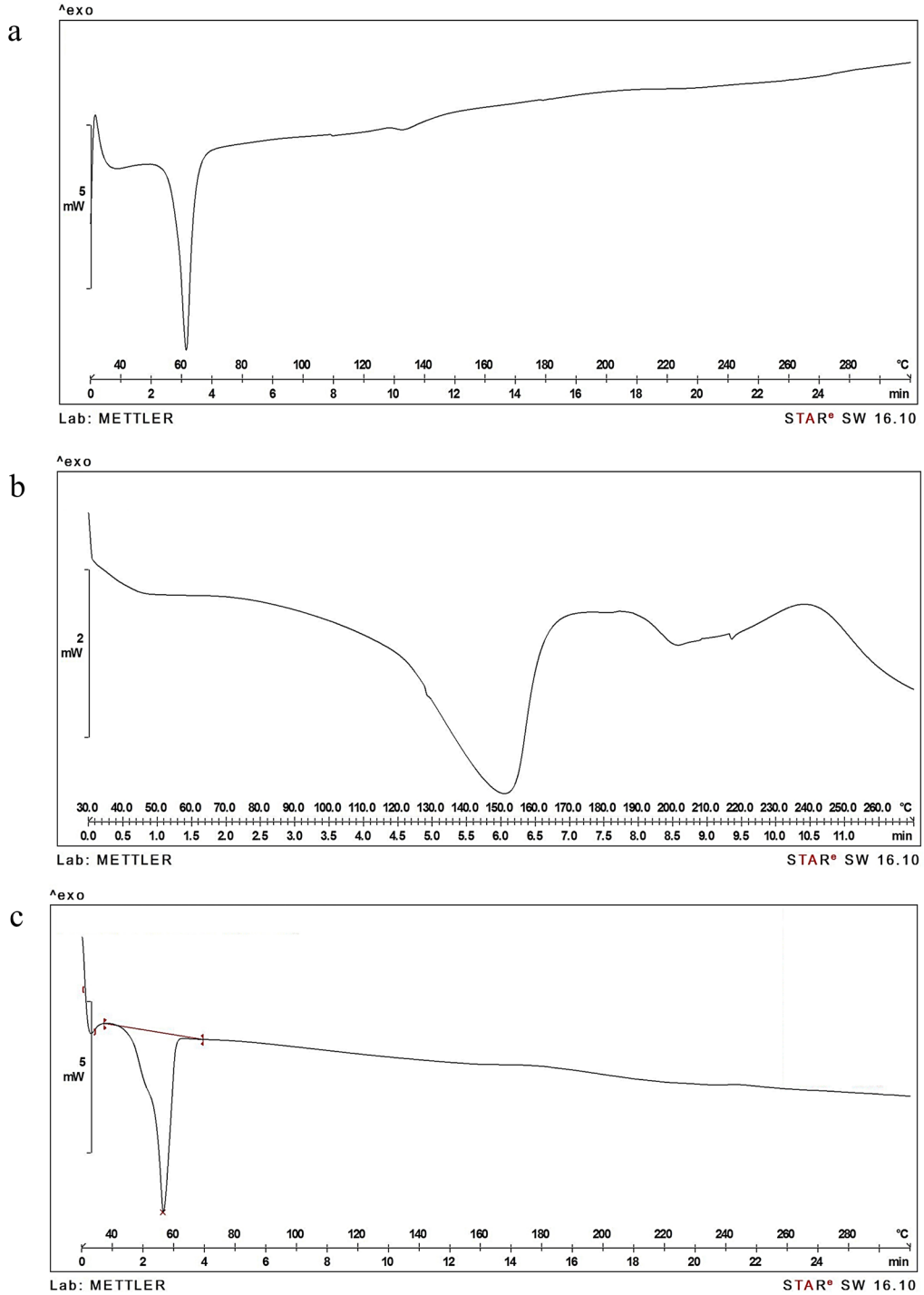

Figure 2: DSC Curves for (a) P407 (b) FA and (c) FA-P407.

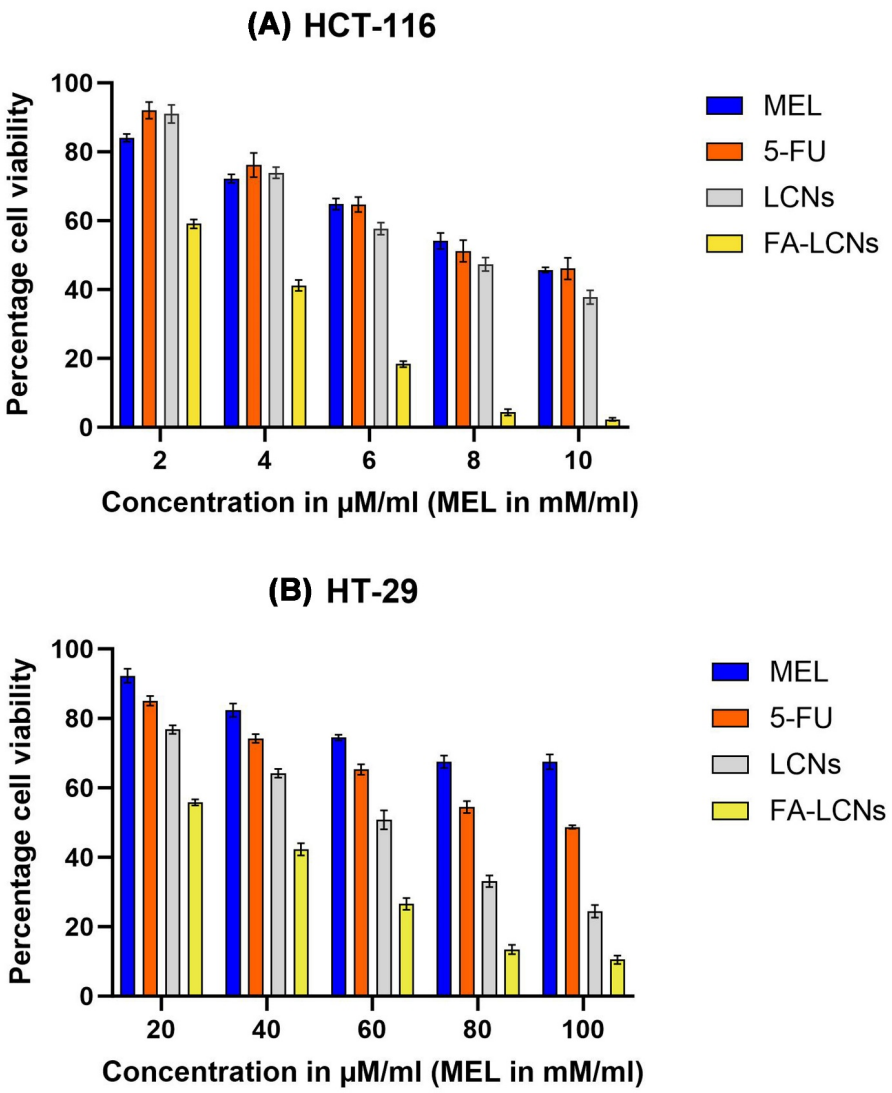

Figure 3: Cytotoxicity assessment of 5FU, MEL, LCNs, FA-LCNs in (A) HCT-116 (B) HT-29 cancer cells (MEL in mM/mL concentration).

LCNs nanostructure promotes improved intracellular drug availability. The bicontinuous lipid architecture of LCNs likely facilitates membrane interaction and endosomal retention, thereby enhancing effective drug concentration within tumor cells. Notably, FA-functionalization resulted in a significantly enhanced cytotoxicity. FA-LCNs reduced the IC_{50} to 3.08 µM, representing approximately a 2.7-fold increase in potency relative to free 5-FU and a 2.5-fold enhancement compared to LCNs. Given the reported overexpression of folate receptors in colorectal carcinoma, ligand-receptor engagement likely accelerates internalization kinetics, increases intracellular drug accumulation, and amplifies downstream apoptotic signaling (Martín-Sabroso *et al.*, 2021; Sudimack and Lee, 2000).

In vitro cytotoxicity of 5-FU, MEL, LCNs, and FA-LCNs against HT-29 cells (Figure(B)) displayed reduced sensitivity to free 5-FU (IC_{50} 94.5 µM), consistent with their documented chemo resistant phenotype. Non-targeted LCNs produced only a modest improvement (IC_{50} 59.57µM), indicating that passive nanoparticle uptake alone was insufficient to substantially overcome intrinsic resistance mechanisms (Gmeiner and Okechukwu, 2023). In contrast, FA-LCNs dramatically enhanced cytotoxic efficacy, lowering the IC_{50} to approximately 28.3 µM. This corresponds to nearly a 3.3-fold improvement compared to free drug and a 2.1-fold enhancement relative to LCNs. The pronounced efficacy in HT-29 cells is particularly noteworthy, as it suggests that active targeting may effectively circumvent reduced drug sensitivity by increasing intracellular drug exposure beyond resistance thresholds. Such findings underscore the functional relevance of receptor-mediated targeting in overcoming partial chemoresistance.

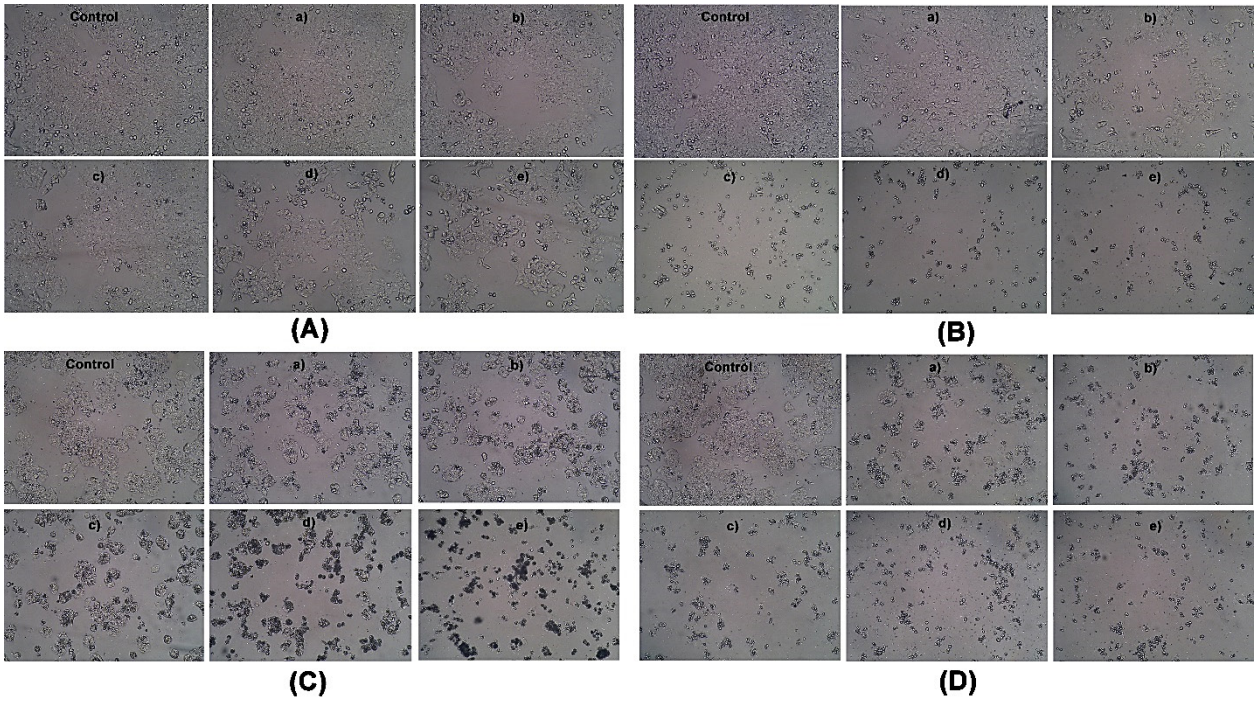

Figure 4: Representative bright-field microscopic images of LCNs(A), FA-LCNs(B), against HCT-116 cancer cells after 72 hr and the concentrations were (a) 2 µM/mL (b) 4 µM/mL (c) 6 µM/mL (d) 8 µM/mL (e) 10 µM/mL. LCNs (C) FA-LCNs (D) against HT-29 cancer cells after 72 hr and the concentrations were (a) 20 µM/mL (b) 40 µM/mL (c) 60 µM/mL (d) 80 µM/mL (e) 100 µM/mL.

CONCLUSION

Comprehensive thermal and spectroscopic analyses confirmed successful covalent conjugation of folic acid to Poloxamer 407. The FA-P407 conjugate maintained structural integrity and provided functional targeting capability for subsequent nanoparticle formulation. Folate-functionalized liquid crystalline nanoparticles demonstrated a marked enhancement in cytotoxic potency, with an approximately 2 to 3-fold reduction in IC_{50} compared to free 5-FU and MEL. This enhancement was particularly pronounced in the relatively chemo resistant HT-29 cells. Collectively, these findings highlight the advantage of folate-mediated targeting and support FA-conjugated liquid crystalline nanoparticles as a promising strategy which may reduce the systemic toxicity of 5-FU therapy suggesting potential utility in overcoming partial drug resistance in colorectal cancer. However further extensive study warranted to support these findings.

ACKNOWLEDGEMENT

This work received financial assistance from the PSG PRIME grant (Project code: PSGCP/B2025/01) sponsored by PSG institute of Medical Science and Research. This work is an integral part of Ph.D research program registered under The Tamil Nadu Dr. M.G.R. Medical University, Chennai. We thank PSG College of Pharmacy, Coimbatore, for providing all the facilities and support

ABBREVIATIONS

CRC: Colorectal cancer; **5-FU:** 5-fluorouracil; **MEL:** Melatonin; **LCNs:** Liquid crystalline nanoparticles; **FA-LCNs:** Folic acid-functionalized Liquid crystalline nanoparticles; **FR:** Folate receptor; **FA:** Folic acid; **GMO:** Glycerol monooleate; **P407:** Poloxamer 407; **FA-P407:** Folic acid-conjugated Poloxamer 407; **DoE:** Design of Experiments; **FT-IR:** Fourier Transform Infrared Spectroscopy; **DSC:** Differential Scanning Calorimetry; **^{1}HNMR:** Proton Nuclear Magnetic Resonance spectroscopy; **CDI:** 1,1′-carbonyldiimidazole; **DMSO:** Dimethyl sulphoxide; **DMEM medium:** Dulbecco's Modified Eagle Medium; **EDTA:** Ethylenediaminetetraacetic acid; **IC_{50}:** Half maximal inhibitory concentration.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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Cite this article: Sathasivam K, Somaskanthan S, Rahman SMH. Synthesis and *in vitro* Evaluation of Folic Acid-Functionalized Liquid Crystalline Nanoparticles for Enhanced Cytotoxicity in HCT-116 and HT-29 Colorectal Cancer Cells. J Young Pharm. 2026;18(2):369-76.