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Research article

Engineering 5-flourouracil and leucovorin-loaded vesicular systems for possible colon specific delivery: *In vitro* evaluation and real time cell assay against HCT-116 colon cell lines

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ABSTRACT

5-flourouracil (5-FU) is typically modulated with leucovorin (LEU) in clinical practice to improve clinical efficacy and patient survival rates. However, this combination has undesirable side effects and makes 5-FU more toxic. Hence, integrating a vesicular system (proniosomes) with another delivery vehicle may improve drug targeting, while resolving the aforementioned drawbacks. This study aimed to engineer 5-FU/LEU proniosomes for possible delivery to the colon. The modified slurry approach was used to create drug-loaded proniosomes (150 mg/9 g of carrier) using both water-soluble (dextrin (DEX) and lactose (LAC)) and insoluble (Neusilin FH_2 (NEU)) carriers. The powdered formulations were filled into Eudragit S100 (10 %)-coated capsules or Eudragit FS 30D capsules for enteric- or colon-specific delivery. In vitro evaluations (flow properties, powder X-ray diffractometry (XRD) analysis, particle size analysis, entrapment efficiency, drug release, scanning electron microscopy (SEM), polydispersity index, Fourier transform infrared spectroscopy (FTIR), and stability studies) were performed on the formulations. An *in vitro* cytotoxicity test [real-time cell assay (RTCA)] against HCT-116 colon cancer cell lines was performed using the optimized formulation. *In vitro* evaluations showed that the nanoparticles had good physicochemical properties. RTCA studies showed sustained cell death with the formulations compared to the pure drug and placebo. The sequential drug release of the colon-targeted capsules containing 5-FU and LEU- loaded proniosomes showed negligible drug release in SGF (pH 1.2) and phosphate buffer solution (pH 6.8) (approximately 11 %) but profound drug release (>80 %) at pH 7.4. Drug-loaded proniosomes engineered for colon targeting (Eudragit S100 (10 %) capsules or Eudragit FS 30D capsules) showed good colon-specific targeting and favorable *in vitro* cytotoxicity profiles.

1. Introduction

Cancer mortality rates are increasing in Africa, making it a new health concern. According to projections, the high population expansion and aging of Africa's population will lead to an increase in almost 70 % of new cancer cases by 2030 [1]. The African context

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is extremely unusual because most cancers coexist with other communicable diseases, such as malaria and Ebola, which are still being tackled. Despite reports indicating that the death rate from cancer in Africa is larger than the sum of the death rates from Acquired Immunodeficiency Syndrome (AIDS), tuberculosis (TB) and malaria, Africa lacks the motivation and dedication required for comprehensive cancer research. While the threat of non-communicable diseases such as cancer is being ignored, our study has focused on communicable diseases such as malaria [2].

Globally, over 600,000 deaths annually are caused by colorectal cancer, which is one of the main cancer-related killers [3]. Owing to the late presentation of colorectal cancer cases (at the metastatic stage) to hospitals, it is also the sixth most prevalent cancer in Africa and has a very high fatality rate. Since 2002, colorectal cancer cases have increased across the whole continent of Africa [4,5]. 5-Fluorouracil (5-FU), a fluoropyrimidine, is the primary medication used to treat CRC. This drug inhibits thymidylate synthase, one of its many cytotoxic mechanisms [6]. 5-FU is currently administered intravenously as a bolus, infusion, or continuous infusion, resulting in unnecessary systemic exposure and the destruction of healthy cells and tissues. Additionally, 5-FU has a relatively short average plasma elimination half-life (16 min), which necessitates frequent injections [7]. Although 5-FU has broad-spectrum anticancer activity, only a relatively small number of patients can benefit from this treatment, necessitating its modulation with leucovorin. Leucovorin, an active metabolite of folic acid, is a coenzyme that is necessary for the production of nucleic acids. Co-administration of leucovorin (LEU) and 5-FU may greatly improve the therapeutic efficacy of 5-FU in cancer patients, although there is an increased risk of toxicity [7,8]. Thus, targeted drug delivery to the disease site may alleviate this challenge.

Organ-based drug delivery is a concept that is used in targeted drug delivery systems. It describes drug delivery at organ and tissue levels. This type of delivery is said to be successful when the drug is localized at the disease site, with low or no exposure of the drug to healthy tissues. In this study, proniosomes were designed to target the colon. Proniosomes are either anhydrous free-flowing formulations or liquid crystals of water-soluble carriers coated with appropriate nonionic surfactants, giving them jelly like consistency. These are also known as “dry niosomes” or “dehydrated niosomes” [9]. They are better than regular niosomes because they are easily reconstituted in an aqueous phase prior to injection or hydrated in body compartments to form niosomal vesicles. The use of only one delivery approach, such as proniosomes, to deliver drugs is often associated with challenges such as rapid systemic clearance, difficulty in producing high concentrations at target locations, and insufficient control over drug release. Thus, integration of two or more delivery approaches into a single system may enhance drug delivery by leveraging the benefits of multiple delivery strategies. These hybrid drug delivery systems also provide advantages such as enhanced therapeutic efficacy, overcoming drug resistance, and controlled and targeted release. In the case of colon targeted delivery, recent studies have focused on the use of conventional niosomes, drug repurposing and the use of microparticulate systems [10,11].

To the best of our knowledge, there is a paucity of information regarding the use of proniosomes integrated into pH-sensitive carriers for targeted drug delivery to the colon. Additionally, the commonly used *in vitro* cytotoxicity assay, the classic colorimetric test [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT), measures cell viability based on the ability of viable cells to convert the yellow MTT dye into purple formazan. This test is limited because it provides a single endpoint measurement based on color change and does not provide real-time information on cell death [8,12]. In this study we designed 5-FU/LEU loaded proniosomes encapsulated in Eudragit S100 (10 %)-coated capsules and/or Eudragit FS 30D capsules for colon-specific delivery. The combination of the unique advantages of proniosomes and Eudragit polymers offers tremendous benefits for targeted drug delivery. A real-time cell assay (RTCA) was used instead of a colorimetric test for the *in vitro* cytotoxicity investigation. We tracked cell death hourly during the entire experiment because the RTCA, as opposed to the MTT test, can capture data in real-time [12]. Therefore, the goal of this study was to investigate the suitability of various carriers (DEX, NEU, and LAC) for the production of proniosomes to encapsulate drug-loaded proniosomes in Eudragit S100 (10 %)-coated capsules and/or Eudragit FS 30D precoated capsules, to carry out *in vitro* evaluations, and to conduct a real-time cell assay (RTCA) against HCT-116 colon cancer cell lines.

Table 1
Formular for the 5-FU/LEU-loaded powdered niosomes or proniosomes.

	P1	P2	P3	P4	P5	P6	P7	P8	P9	P10	P11	P12	P13	P14	P15	P16
Tw 60(mg)	–	–	–	–	–	–	210	150	150	150	150	–	–	150	150	–
Sp 60 (mg)	150	150	150	210	–	–	–	–	–	–	–	150	150	–	–	150
Tw 40(mg)	–	–	–	–	–	150	–	–	–	–	–	–	–	–	–	–
Sp 40(mg)	–	–	–	–	210	–	–	–	–	–	–	–	–	–	–	–
SOL (mg)	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30	30
CHL (mg)	150	150	150	90	90	150	90	150	150	150	150	150	150	150	150	150
NEU (g)	9	–	–	–	–	–	–	–	–	9	–	–	9	9	–	–
LAC (g)	–	9	–	–	–	–	–	–	9	–	9	9	–	–	–	–
DEX (g)	–	–	9	9	9	9	9	9	–	–	–	–	–	–	9	9
5-FU(mg)	150	150	150	150	150	150	150	150	150	150	–	–	–	–	–	–
LEU (mg)	–	–	–	–	–	–	–	–	–	–	150	150	150	150	150	150

*5-FU -5-flourouracil, LEU- Leucovorin, Sp 60 – Span 60, Span 40 – Span 40, Tween 40 – Tween 40, Tween 60 – Tween 60, CHL- Cholesterol, NEU –Neusilin FH_2®, SOL- Soluplus®,LAC – Lactose, DEX – Dextrin.

2. Materials and methods

2.1. Materials

The materials which include 5-fluorouracil, Tween® 40, Tween® 60, Span® 40 and Span® 60 were obtained from Aladdin China. Leucovorin was a gift sample from Merck, Switzerland; Span® 20 and cholesterol were obtained from Abcam, United Kingdom; Pluronic® F68 was obtained from Life Technologies USA, Neusilin® FH_2 and Soluplus® were gift samples from BASF Pharma, Germany. Dextrin (BDH England), lactose, ethanol, hydrochloric acid, polyethylene glycol (PEG) 400, acetone and methanol were obtained from Sigma Aldrich, Germany, while Eudragit S100 and Eudragit FD 30S were gift samples from Evonik, Germany. HCT-116 colon cancer cell line was obtained from ATCC USA.

2.2. Formulation of 5 fluorouracil and leucovorin loaded powdered niosomes/proniosomes

Proniosomes (P1 – P16) were produced using the slurry method (Table 1). Accurately weighed amounts of surfactants and cholesterol were dissolved in 5 ml of ethanol. 5-FU or LEU (150 mg) was dissolved in a mixture of ethanol and distilled water (2:8) and added to this mixture (Table 1). This mixture was transferred into a 100 ml beaker, and Neusilin FH_2 (NEU) was added to form slurry. The beaker was placed on a magnetic stirrer, the organic solvent was evaporated at a temperature of 40 ± 2 °C, and the slurry was further dried in a desiccator at room temperature until a dry powder was formed. This procedure was repeated using water-soluble carriers, dextrin (DEX) powder and lactose (LAC) granules (maize starch 5 % w/v was used to granulate native lactose). Soluplus® (SOL) (10 mg/ml) was used as a binder to improve the flow of the Neusilin-based formulations [9,13].

2.3. Granulation properties of the proniosomes

The powdered proniosomes were weighed in 4 g portions. Each one was added to a measuring cylinder with a capacity of 50 ml. The cylinder was dropped on a wooden platform three times at intervals of 2 s from a height of one inch, and the bulk volume occupied by the powder was measured. The tapped volume was determined by tapping on the wooden platform until the volume of the powder remained constant. The tapped density and bulk density were calculated using equations (1) and (2) respectively. Other granulation parameters such as flow rate, angle of repose, Hausner's quotient and Carr's compressibility index were also determined.

$$Bulk\ density = \frac{Mass\ (gram)}{Bulk\ volume\ (cm^3)} \tag{1}$$

$$Tapped\ density = \frac{Mass\ (g)}{Tapped\ volume(cm^3)} \tag{2}$$

2.4. Particle size analysis and polydispersity index of powdered formulations

Accurately weighed amount of proniosomal powder was dispersed in distilled water with vigorous shaking and thereafter filtered. Transparent aqueous dispersions of each of the 5- FU/LEU-loaded niosomes were diluted hundredfold with distilled water. The droplet size and polydispersity index were determined using a zeta sizer (Malvern Instruments, U.K) at a light scattering angle of 90° [14].

2.5. Encapsulation efficiency and drug loading capacity of powdered niosomes

The drug content was done by dispersing 100 mg of the powdered noisome in 10 ml of phosphate buffer (pH 7.4) with mild shaking after which 30 ml of methanol was added. The mixture was sonicated for 20 min and filtered via filter paper. After appropriate dilutions, the drug content was determined via the UV spectrophotometer at the wavelength of the drugs (266 nm for 5-FU and 286 nm for LEU). The entrapment efficiency was determined by hydrating 100 mg of powdered niosomes in 5 ml of phosphate buffer (pH 7.4). A 1 ml volume was centrifuged at 12,000 rpm for 5 min. The supernatant was then analyzed for the free drug. The entrapment efficiency and loading capacity were calculated according to equations (3) and (4) respectively:

$$Entrapment\ efficiency = \frac{Total\ drug\ loaded - Drug\ in\ supernatant}{Total\ drug\ loaded}\ x\ 100\ \% \tag{3}$$

$$Drug\ loading\ capacity = \frac{Amount\ of\ drug\ entrapped}{Total\ weight\ of\ the\ nanoparticles}\ x\ 100\ \% \tag{4}$$

2.6. Morphology of powder via scanning electron microscopy (SEM)

The surface morphology of the carriers (DEX, LAC and NEU) and the proniosomes were investigated using scanning electron microscope (Hitachi Japan, Model 3400 N). The samples were fixed on a glass stud, coated with thin layers of gold particles and the SEM images were recorded at a voltage of 15 Kv and a magnification of 500x

2.7. Powder X-ray diffractometry (XRD) analysis

The X-ray diffractometry (XRD) of the pure drug, the proniosomes and the carriers (powder) were determined using the X-ray diffractometer (Rigaku Miniflex, Japan). The analyzed material was finely ground and homogenized, after which it was compressed in the sample holder to create a smooth surface and then mounted on the sample stage in the XRD cabinet. The sample was analyzed using the reflection – transmission spinner stage using theta-theta settings. The intensity of diffracted X-ray was continuously recorded as the sample and detector rotate through their respective angles (4°–75°) at 8.67 s per step. The current and voltage employed were 40 mA and 45 VA respectively. A peak in intensity occurred when the material containing lattice planes with d-spacing appropriately diffract X-rays at that value of θ [15].

2.8. Drug–excipient compatibility studies via FTIR

The infra-red spectra of the carriers and drug loaded formulations were determined using FTIR spectrophotometer. The conventional KBr pellet method was used with scanning done between 4000 and 500 cm^{-1}.

2.9. Coating of capsules and the use of pre coated Eudragit FD30S capsules for colon delivery

The HPMC capsules were coated with 10 % Eudragit S100 by dip coating method. The Eudragit S100 (10 g) was dissolved in 100 ml of methanol and acetone at the ratio of 20:14.5 to get a uniform solution of 10 %w/v. Polyethylene (PEG) 400 at 3 % was added [16]. Coating was done by dipping the capsules into the coating solutions for 1 min and dried at room temperature. This was done till a final weight gain of 60–65 % was obtained. The capsules were then allowed to dry overnight at room temperature and stored for further studies. Pre coated Eudragit FD30S® capsules (EVONIK, Germany) were also employed for the colon delivery of the proniosomes. The proniosomes (600 mg) equivalent to 10 mg of 5-FU/LEU was filled into these capsules and stored till further use.

2.10. Drug release studies/dissolution studies for coated capsules

The *in vitro* drug release study was done using the rotating basket method with the coated capsules trapped in the basket. The dissolution studies was done sequentially at intervals in simulated gastric fluid (SGF, pH 1.2), and phosphate buffer (pH 6.8 & 7.4) for a period of 24 h. The temperature and speed were maintained at 37 ± 1 °C and 100 rpm respectively. At predetermined time intervals, 5 ml was withdrawn and replaced with a corresponding fresh medium to maintain constant volume. Each of the samples gotten for each drug was then assayed using the UV-spectrophotometer (Spectrum Lab, UK) wavelengths of 266 nm and 286 nm for 5-FU and LEU respectively. All assays were done in triplicate.

2.11. In vitro cytotoxicity studies [(real time cell assay (RTCA)]

HCT-116 colon cancer cell lines were stored in the vapor phase of a liquid nitrogen chamber until use. The cells were then rapidly thawed at 37 °C in water and subsequently suspended in 5 ml of complete medium supplemented with 10 % fetal bovine serum (FBS). The suspended cells were incubated at 37 °C and 5 % CO_2 until passaging was achieved. The cells were passaged 3 times before using in the cytotoxicity test. HCT-116 cells were seeded per well in a 16-well RTCA culture plate overnight to allow cell adherence. Solutions containing 50 μM 5-FU and/or 10 μM LEU from the optimized formulation, as well as the pure drugs, were added to the plates and

Table 2
Granule properties of 5-FU and LEU-loaded proniosomes.

Batch no	BD (g/cm^3)	TD (g/cm^3)	AR (°)	FR (g/s)	HQ	CI (%)
P1	0.77 ± 0.01	0.99 ± 0.01	17.06 ± 1.14	4.19 ± 0.06	1.28 ± 0.03	21.92 ± 1.63
P2	0.65 ± 0.01	0.55 ± 0.00	15.19 ± 0.93	3.61 ± 0.16	1.19 ± 0.02	16.36 ± 1.12
P3	0.77 ± 0.01	0.87 ± 0.01	16.19 ± 0.77	4.00 ± 0.16	1.13 ± 0.01	11.28 ± 1.11
P4	0.69 ± 0.00	0.77 ± 0.01	22.91 ± 0.41	3.95 ± 0.04	1.12 ± 0.01	10.66 ± 1.05
P5	0.69 ± 0.00	0.79 ± 0.01	16.62 ± 0.70	3.98 ± 0.11	1.15 ± 0.01	13.22 ± 0.81
P6	0.69 ± 0.00	0.79 ± 0.01	14.42 ± 0.27	4.12 ± 0.23	1.15 ± 0.01	12.97 ± 0.71
P7	0.69 ± 0.01	0.84 ± 0.01	11.46 ± 0.22	4.07 ± 0.13	1.21 ± 0.00	17.34 ± 0.14
P8	0.72 ± 0.01	0.84 ± 0.01	19.42 ± 1.15	4.30 ± 0.55	1.17 ± 0.00	14.37 ± 0.12
P9	0.52 ± 0.00	0.71 ± 0.01	20.09 ± 0.68	3.16 ± 0.12	1.35 ± 0.02	26.20 ± 0.93
P10	0.79 ± 0.01	0.95 ± 0.00	16.05 ± 0.29	3.98 ± 0.02	1.19 ± 0.00	16.22 ± 0.31
P11	0.52 ± 0.00	0.69 ± 0.01	22.45 ± 0.70	2.48 ± 0.65	1.33 ± 0.02	24.78 ± 0.94
P12	0.59 ± 0.00	0.77 ± 0.00	20.27 ± 0.84	3.24 ± 0.12	1.31 ± 0.01	23.47 ± 0.53
P13	0.87 ± 0.00	1.10 ± 0.01	18.56 ± 1.16	4.33 ± 0.27	1.27 ± 0.01	21.30 ± 0.62
P14	0.84 ± 0.01	1.06 ± 0.01	16.49 ± 0.57	4.51 ± 0.28	1.26 ± 0.02	20.97 ± 1.54
P15	0.69 ± 0.01	0.87 ± 0.00	17.64 ± 0.41	4.14 ± 0.17	1.25 ± 0.01	20.22 ± 0.65
P16	0.71 ± 0.00	0.87 ± 0.00	16.16 ± 0.39	4.56 ± 0.08	1.32 ± 0.14	23.46 ± 7.41

*P1 –P10 – 5-FU-loaded proniosomes, P11 – P16 –LEU – loaded proniosomes, BD – bulk density, TD-tapped density, AR – angle of repose, FR – flow rate, HQ- Hausner's quotient, CI – Carr's compressibility index.

incubated at 37 °C in a 5 % CO_2 incubator. Real –time cell assay was monitored on RTCA software for 24 h and the results were collected and analyzed [12,17].

2.12. Statistical analysis

The data obtained were analyzed using SPSS Version 26.0 (SPSS Inc. Chicago, IL, US). Values were presented as mean ± SD (standard deviation). Means were compared using one way ANOVA and $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Granule properties of 5-FU/LEU-loaded powdered niosomes/proniosomes

Table 2 shows results of the Hausner's quotient (HQ), Carr's compressibility index (CI), angle of repose and flow rate values of 5-FU and LEU loaded proniosomes respectively. The granulation properties generally indicate fair/passable flow behavior. Angle of repose is a measure of the internal friction or cohesion of the particles. It is high if cohesive and other forces are high and vice versa. Generally, if the angle exceeds 50°, the powder will not flow satisfactorily while materials having values near the minimum, circa 25°, flow easily and well [18,19]. It should be noted that three different carriers (DEX, LAC and NEU) were used. The DEX-based proniosomes showed the best flow under gravity followed by NEU - based proniosomes and then the LAC-based proniosomes. Native LAC and NEU had

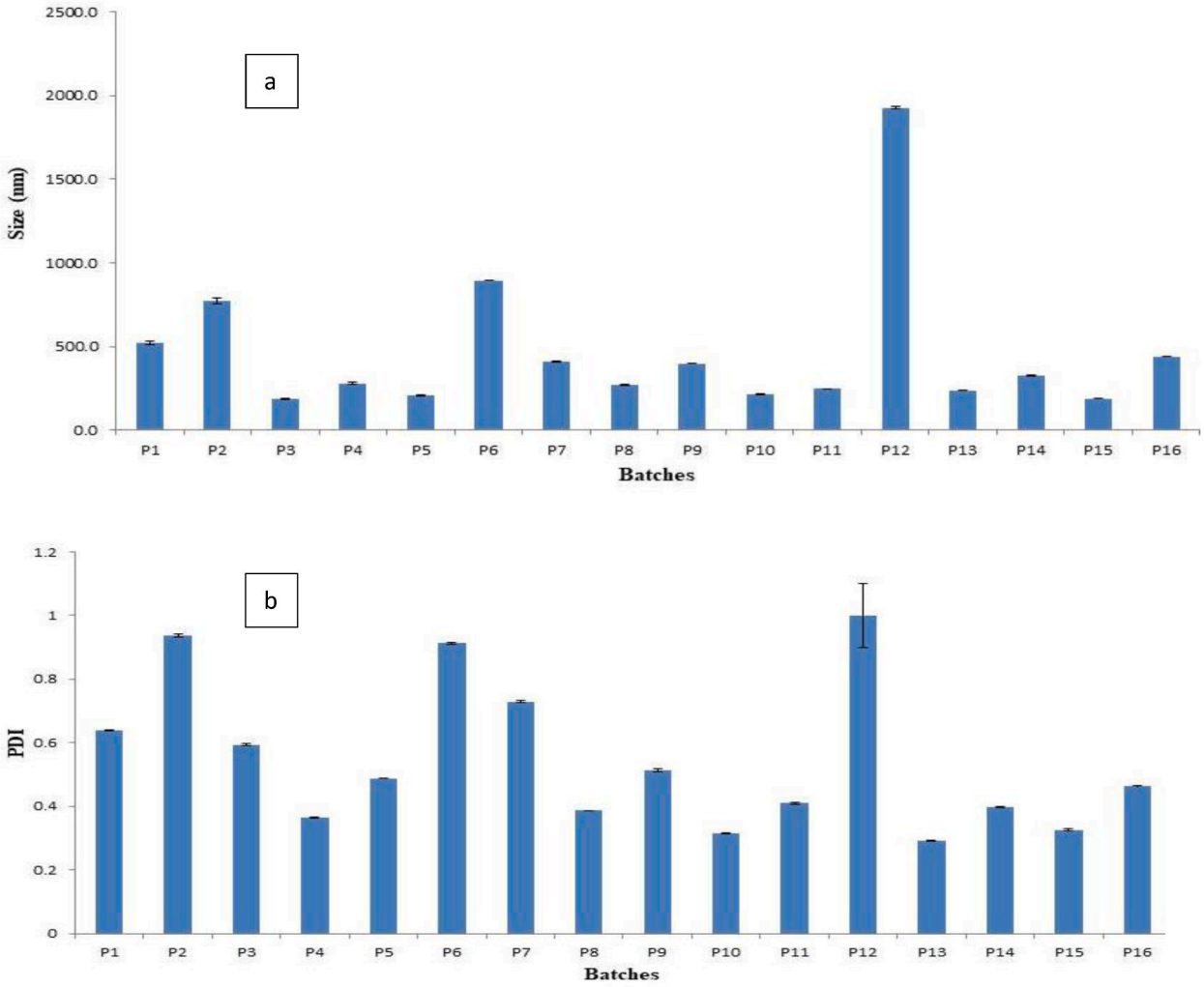


Fig. 1. a. Particle size of 5-FU/LEU-loaded powdered niosomes/proniosomes, b. Polydispersity index (PDI) of 5-FU/LEU-loaded powdered niosomes/proniosomes c. Size distribution of 5-FU-loaded dextrin-, lactose- and Neusilin-based proniosomes *P1 –P10 – 5-FU-loaded proniosomes, P11 – P16 –LEU – loaded proniosomes, P1, P10, P13, P14 – Neusilin-based proniosomes, P2, P9, P11, P12 – lactose-based proniosomes, P3 – P8, P15, P16 – dextrin-based proniosomes * Dex – dextrin-based, Lac-lactose-based, Neu – Neusilin-based

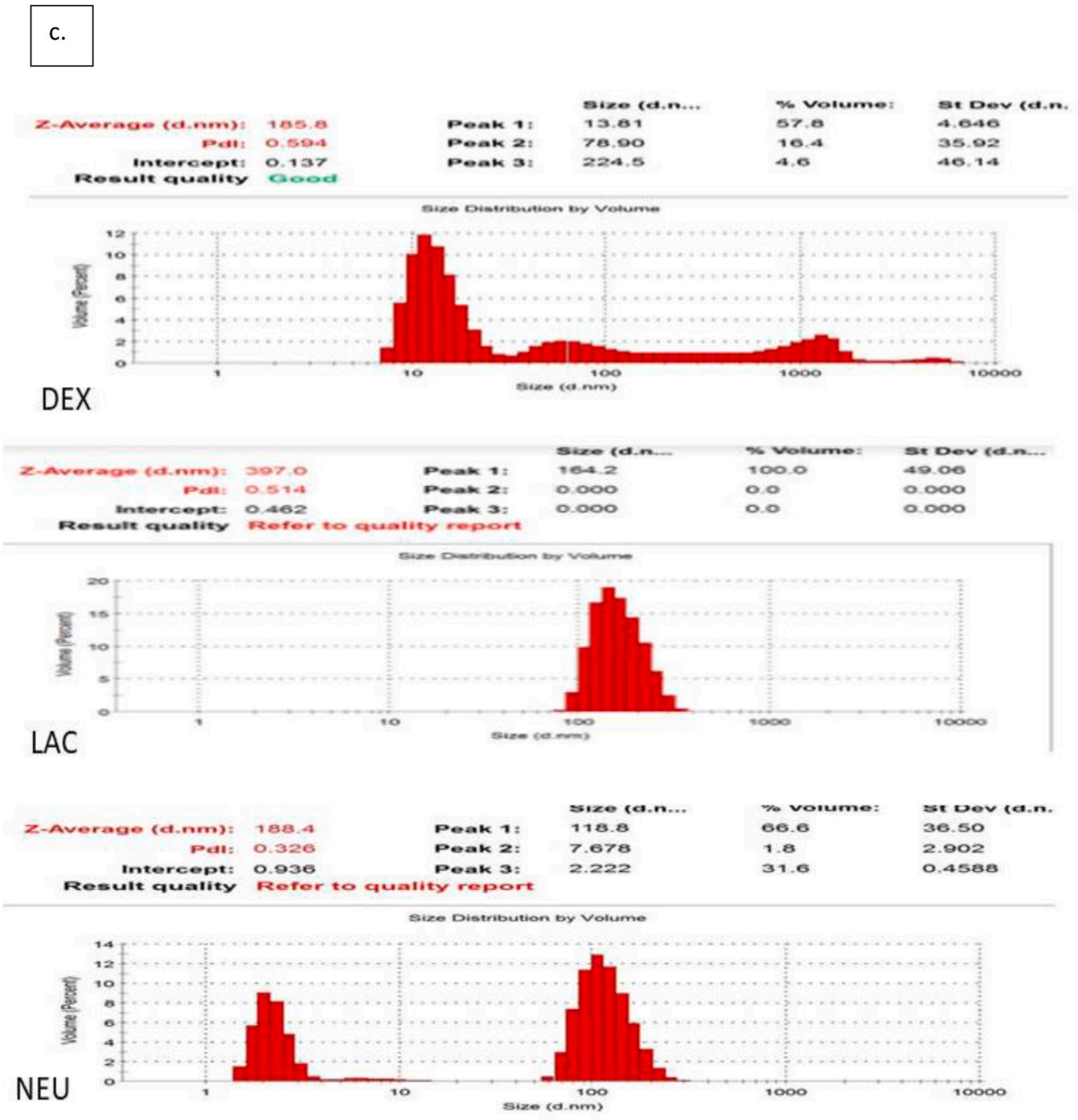


Fig. 1. (*continued*).

intrinsic poor flow, therefore they were both subjected to some form of granulation to aid flow. Lactose powder was granulated using 5 % w/v maize starch prior to its use in the formulation of proniosomes, while the NEU proniosomes was granulated with aqueous dispersion of Soluplus (3 % w/v). Afterwards flow was improved, such that the granules were sufficient to flow into the capsules. Wet granulation is often associated with the formation of liquid bridges between particles, with the tensile strength of these bonds increasing as the quantity of granulating fluid increases. Thus in the process of drying, formation of interparticulate bonds due to the fusion or recrystallization and curing of the binding agent takes place [20,21]. Based on the angle of repose, Hausner's quotient and Carr's compressibility index results, all of the batches demonstrated acceptable granulation properties. It has been reported that HQ values less than 1.25 indicate good flow, while values greater than 1.25 indicate poor flow. HQ values between 1.25 and 1.5 require addition of a glidant to improve flow. The flow scale of powder and granulations have been described as excellent, for values of CI within 5–15 %, good for 12–16 % and fair to passable for 18–21 %, while between 23 and 35 % are said to be poor, 33–38 % are very poor and values $>$ 40 % are extremely poor [22]. HQ and CI are useful indices for assessing drug powder and granule flow properties.

These flow indices are not individually sufficient to confirm powder or granulation flowability. For instance, a powder could have high Carr's compressibility index and still flow well under gravity and vice versa. Therefore it has been reported that no one test or index could reliably reflect powder flow ability. The general flow behavior of the proniosomes was sufficient to guarantee free and easy flow into the capsules, however for the formation of tablet, glidants may be added to facilitate flow.

3.2. Particle size/polydispersity index (PDI) of 5-FU/LEU-loaded powdered niosomes/proniosomes

The particle sizes of the hydrated 5-FU-loaded proniosomes ranged from 185.8 ± 4.08 nm to 893.5 ± 2.45 nm, while the particle size of the LEU-loaded proniosomes ranged from 188.4 ± 0.33 nm to 1927 ± 5.71 nm (Fig. 1a). The DEX-based formulations loaded with 5-FU (P3, P4, P5, P7, and P8) generally had significantly smaller particle sizes ($p < 0.05$) than either the NEU- or LAC-based proniosomes, with the exception of P6, which had a large particle size of 893.5 ± 2.45 nm (insert ± PDI). The 5-FU-loaded NEU or LAC-based proniosomes containing Tweens (P9, P10) showed smaller particle sizes ($p < 0.05$) compared to those containing Spans (P1, P2). This trend was also observed for LAC- or DEX-based proniosomes loaded with LEU; however, the reverse was observed in LEU-loaded NEU formulations. The smallest particle sizes of 185.8 nm and 188.4 nm were observed with the dextrin-based formulation containing Span 60 and loaded with 5-FU (P3) and LEU containing Tween 60 (P15) respectively. The type and quantity of surfactants and the concentration of cholesterol are known factors that influence the particle sizes of vesicles [2]. In our study, when the quantity of Span 60 increased from 50 % (P3) to 70 % (P4), the particle size increased significantly ($p < 0.05$) from 185.8 nm to 276.4 nm. This pattern (increased particle size) was also observed when Tween 60 concentration was increased from 50 % (P8) to 70 % (P7). Our results are consistent with those of previous studies [23–26,27]. Vesicle size is critical for nanovesicular carriers because it influences stability, entrapment efficiency, bioavailability, and bio-distribution. Therefore, most of the 5-FU/LEU-loaded proniosomes had particle sizes <500 nm. The various carriers used may also have influenced the particle size results. Smaller sizes were observed in the majority of the DEX-based proniosomes. This was consistent with a previous study that compared LAC and maltodextrin as carriers for the production of resveratrol-loaded proniosomes, where it was found that maltodextrin-based proniosomes had smaller particle sizes than LAC-based proniosomes [28]. DEX is a hydrophilic polymer containing large amounts of hydroxyl groups that can be easily modified [29]. The hydrophilicity and potential to interact with the lipid portion of the formulations may have led to smaller particle sizes. All carriers employed in the formation of proniosomes have the potential to produce nanosized vesicles upon hydration *in vivo*. However, DEX-based proniosomes had the smallest particle sizes. The PDI ranged from 0.292 to 1.0 (Fig. 1b) for the 5-FU- and LEU-loaded proniosmes. PDI is a measure of the size distribution of vesicles in the formulations. Most of the DEX-based formulations had the lowest PDI, followed by NEU and LAC-based formulations (Fig. 1c). When the PDI is closer to zero, it indicates monodispersity and homogeneity, while PDI closer to one indicates polydispersity and heterogeneity [26]. Therefore, all formulations that had PDI less than 0.4 can be said to be monodispersed and homogeneous.

3.3. Encapsulation efficiency and drug loading capacity of 5-FU/LEU-loaded powdered niosomes/proniosomes

The encapsulation efficiency (EE) of the 5-FU-loaded proniosomes ranged from 59.28 % to 87.05 %, while the LEU-loaded proniosomes ranged from 78.31 % to 88.73 % (Fig. 2). Most of the DEX-based proniosomes had EE > 80 % ($p < 0.05$) compared to NEU or LAC-based proniosomes which had EE mostly >70 %. Generally, proniosomes containing surfactants with long alkyl chain are known to have higher entrapment efficiency. Conversely, the ratio of the amount of drug that is trapped to the total weight of polymers used in the formulation is known as the drug loading capacity (DLC) [14]. The DLC of the 5-FU/LEU proniosomes ranged from 0.93 to 1.42 % (Table 3). Significant or discernible differences ($p < 0.05$) in DLC were seen within some of the batches. For the 5-FU–loaded formulations, the DEX carrier (seen in P7) seemed to have impacted higher drug loading capacity ($p < 0.05$) compared to the LAC or NEU carrier (P1, P9, P10). On the other hand, for the LEU-loaded proniosomes, the batches containing NEU carrier showed higher DLC than the DEX and LAC carrier. Sammour et al. reported high EE in respective of the carriers utilized in the formulation of proniosomes

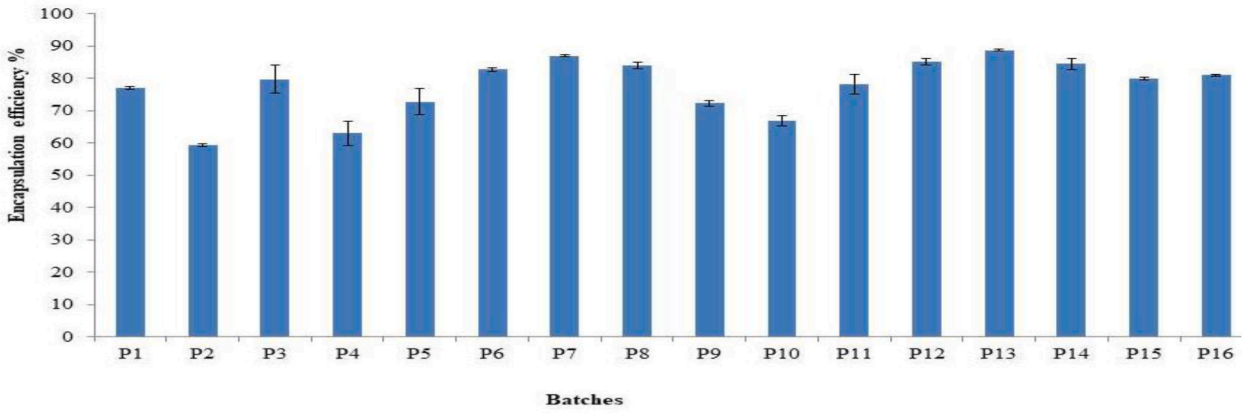


Fig. 2. Encapsulation efficiency (EE) of 5-FU/LEU-loaded powdered niosomes/proniosomes.

Table 3
Drug loading capacity of 5-FU/LEU-loaded proniosomes.

Batches	EE (%)	DLC (%)
P1	77.00 ± 0.52	1.22 ± 0.01
P2	59.28 ± 0.42	0.94 ± 0.01
P3	79.80 ± 4.29	1.26 ± 0.07
P4	63.06 ± 3.71	1.00 ± 0.06
P5	72.73 ± 4.06	1.15 ± 0.06
P6	82.79 ± 0.61	1.31 ± 0.01
P7	87.05 ± 0.43	1.38 ± 0.01
P8	84.00 ± 1.00	1.33 ± 0.02
P9	72.30 ± 0.81	1.14 ± 0.01
P10	66.87 ± 1.50	1.06 ± 0.02
P11	78.31 ± 3.09	1.24 ± 0.05
P12	85.09 ± 1.03	1.35 ± 0.02
P13	88.73 ± 0.22	1.40 ± 0.00
P14	84.45 ± 1.85	1.34 ± 0.03
P15	79.84 ± 0.41	1.26 ± 0.01
P16	81.02 ± 0.33	1.28 ± 0.01

*P1 –P10 – 5-FU-loaded proniosomes, P11 – P16 – LEU – loaded proniosomes, P1, P10, P13, P14 – Neusilin-based proniosomes, P2, P9, P11, P12 – lactose-based proniosomes, P3 – P8, P15, P16 – dextrin-based proniosomes.

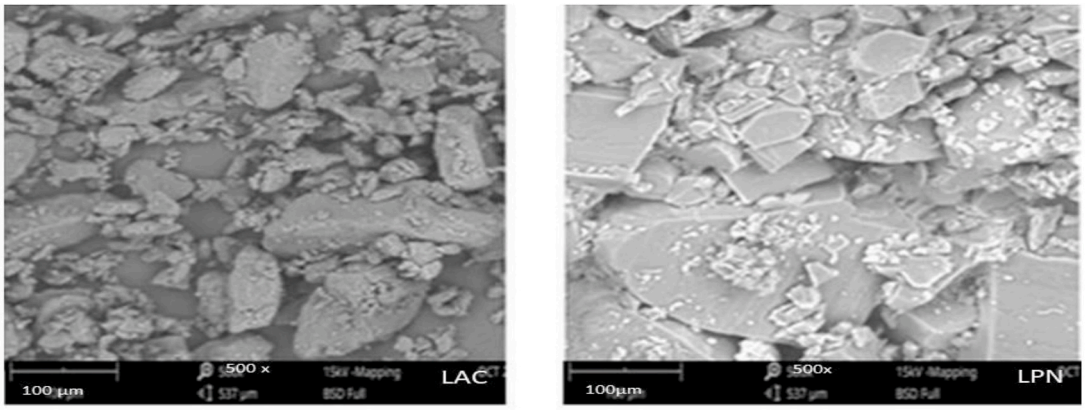
nevertheless he explained the contribution of cholesterol as one of the reasons for good entrapment [26]. Our study showed similarities to their findings, as good entrapment was seen with all the carriers; however, DEX-loaded proniosomes showed higher EE. The ease of hydration with DEX compared to NEU and LAC may have contributed to the higher EE and DLC seen. Recall that the carriers imparts higher surface area and flexibility, thus more drugs are likely to be entrapped within the proniosomes compared to the conventional niosomes [9]. In summary, the DEX and NEU powder seemed to have imparted greater surface area compared to LAC.

3.4. Scanning electron microscopy (SEM) of 5-FU/LEU-loaded powdered niosomes/proniosome

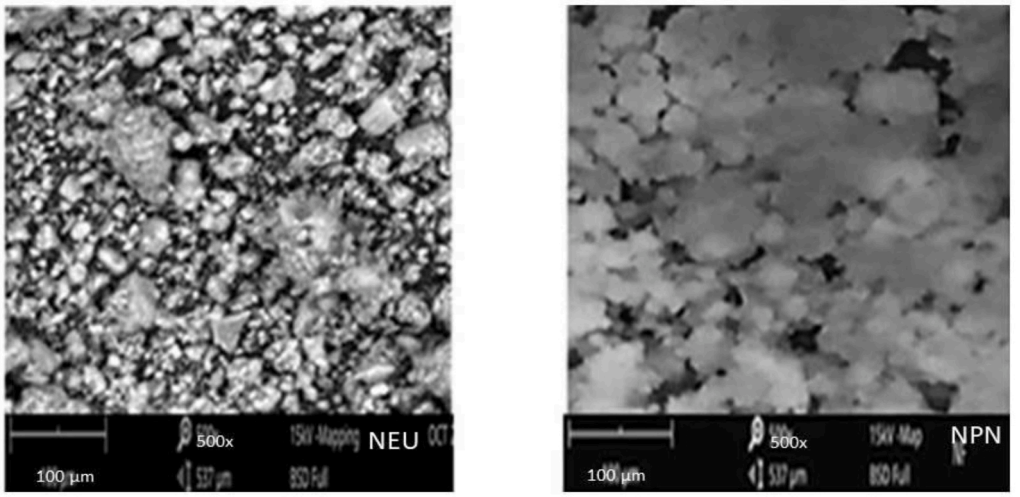
The SEM was used to study the morphology of the proniosomes. The SEM results are presented in Fig. 3a–c. The process of producing proniosomes allows the carriers to be covered with thin film of surfactants [13]. In our results it is seen that the particles of the DEX or LAC or NEU were coated with the surfactant and lipid mix. Nasr and his team [25] reported smoother surfaces with the LAC-based proniosomes when compared to the LAC. They explained that the smooth surfaces observed were due to the packing effects of the surfactants used, and further buttressed that the thickness observed was a result of the deposition of the surfactant – cholesterol mixture at various points of deep invaginations on lactose [25]. Some researchers have also reported aggregates of individual nanoparticles that appeared smooth surfaced and nearly spherical to polyhedral in shape for maltodextrin-based formulations loaded with resveratrol [28]. They observed cracked or flake-like structures with sharp edges in LAC-based formulations. Based on the SEM results, proniosomal formulations with thicker surfaces depicting the carrier powders were coated with the surfactant and cholesterol mix. The DEX-loaded proniosomes also appeared as aggregates of individual particles that appeared porous, whereas the LAC- and NEU-based proniosomes appeared cracked and flaky. This finding is consistent with those reported by Shruthi et al. [28]. In the course of proniosome preparation, the solvent evaporates allowing the dissolved or dispersed solid carrier molecules as well as the surfactant mix to interact at molecular levels. Consequently, some of the fine crystalline structures of the carrier powder are lost; making their surfaces appear coated and presenting with granular surfaces [26,30].

3.5. Xray diffraction studies (XRD) on powdered niosomes/proniosomes

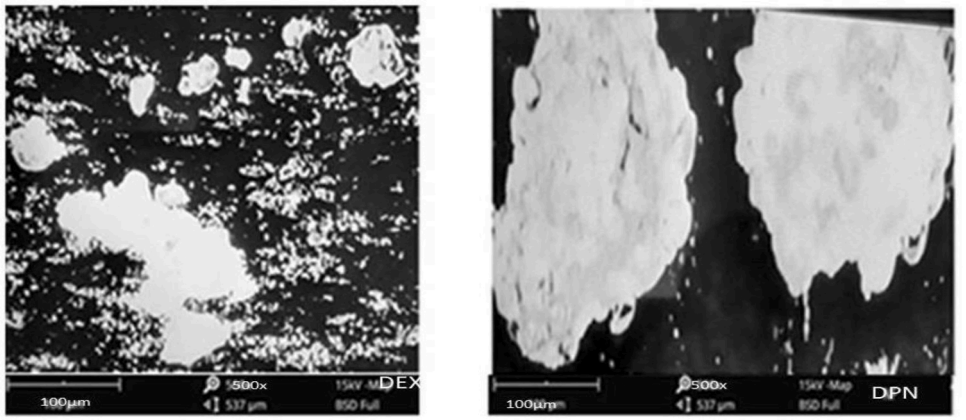
Xray diffraction studies were done to properly assess the degree of crystallinity of the proniosomes and its constituents**.** The results are presented in Fig. 4(a–d). The pure drugs – 5-FU and LEU showed sharp and high peaks intensity of 10000 & 8000 at angle (2θ) of 29° and 38° respectively. Consequently, less intense peaks were also seen at various angles (2θ) on the diffractogram of either the proniosomal formulations or the carriers. These sharp peaks signify that the pure drug samples were crystalline in nature. In XRD studies, crystallinity is usually determined by comparing representative peak height in the diffraction pattern of the test samples with that of reference [13]. Lactose monohydrate has been reported to be crystalline with intense peaks at angle (2θ) 20°, while dextrin diffraction pattern shows no definite peak, so it is described as amorphous [31,32]. In our study, it is observed that Lactose showed maximum intensity at angle (2θ) 20° while dextrin showed no definite peaks. Vadher and his team studied the diffraction patterns of Neusilin US_2 when formulating co-grinded mixtures of aceclofenac, and reported no peaks with the XRD studies, confirming the amorphous nature of Neusilin US_2 [33]. Though another grade of Neusilin (Neusilin FH_2) was used in our study, similar findings were observed. In this study, XRD results for lactose, dextrin and Neusilin FH_2 are consistent with the reports of the researchers previously mentioned [33]. All the proniosomal formulations exhibited lower intensity compared to the pure drugs. The DEX-based proniosomes had the least intensities, followed by NEU and LAC-based proniosomes. The DEX-based proniosomes can be said to be amorphous while the other proniosomal formulations containing Neusilin and lactose can be referred to as semi-crystalline. Consequently, the



a: SEM Images for Lactose (LAC) and Lactose-based proniosomes (LPN) magnification (Mag) = 500 X



b: SEM Images for Neusilin FH_2 (NEU) and Neusilin-based proniosomes (NPN), Mag = 500 X



c: SEM images for dextrin (DEX) and dextrin-based proniosomes (DPN), Mag = 500X

Fig. 3. aSEM Images for Lactose (LAC) and Lactose-based proniosomes (LPN) magnification (Mag) = 500 X
Fig. 3b: SEM Images for Neusilin FH_2 (NEU) and Neusilin-based proniosomes (NPN), Mag = 500 X
Fig. 3c: SEM images for dextrin (DEX) and dextrin-based proniosomes (DPN), Mag = 500X

a

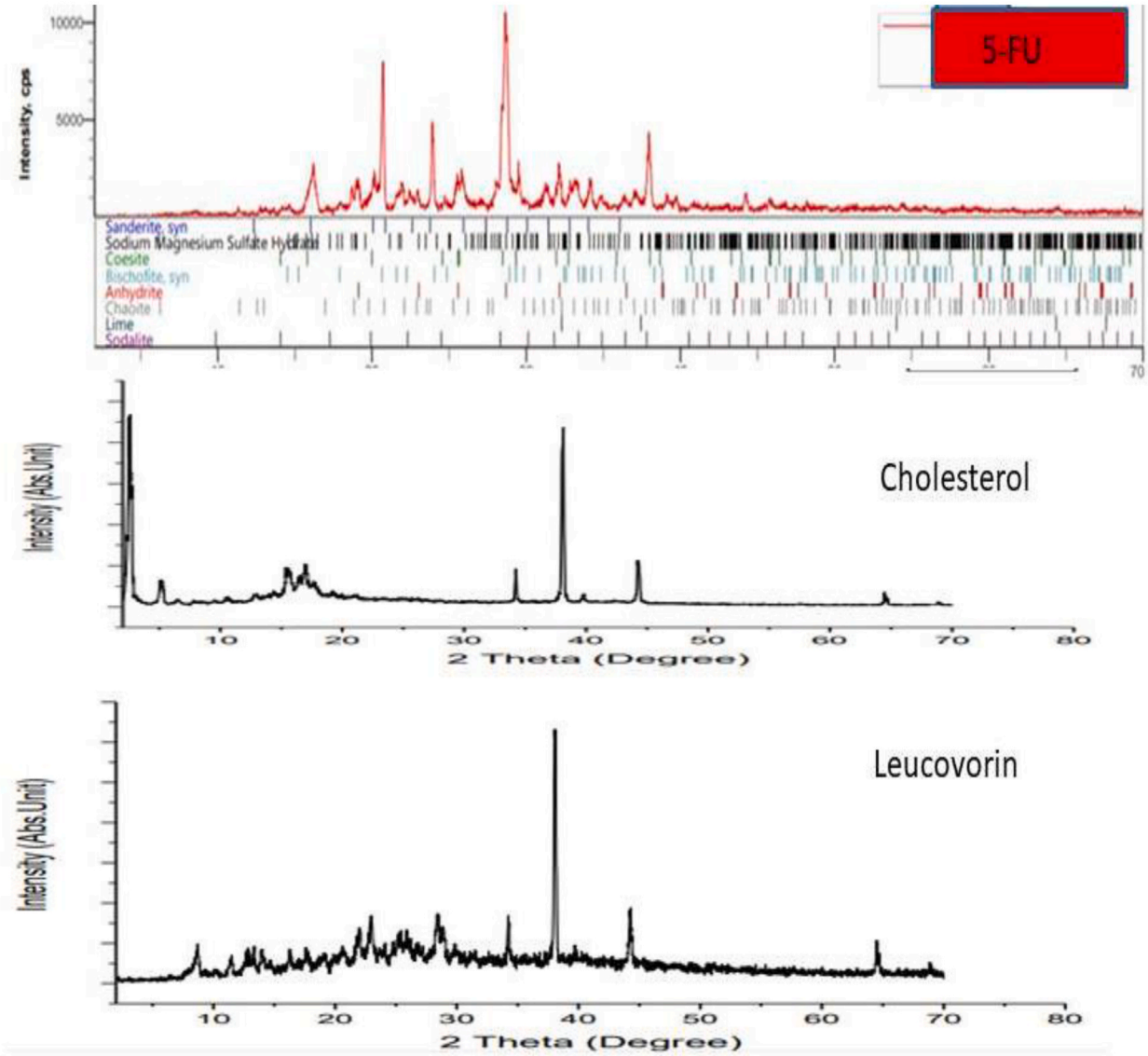


Fig. 4. XRD results of 5- FU, LEU, cholesterol and proniosomal formulations

proniosomes had the ability to impart amorphicity on the drugs of study [26]. This was also reported in a previous study on letrozole proniosomes which showed the disappearance of crystallinity of letrozole when incorporated into the proniosomal carrier [34]. A relationship exists between the degree of crystallinity or amorphicity and solubility of the proniosomes on hydration. Amorphous materials are associated with higher solubility and vice versa with crystalline materials [35]. This implies that the DEX-based formulations are expected to exhibit better solubility profile on hydration *in situ*. Solubility of the carriers may limit or enhance the dissolution and bioavailability of the drug after oral administration.

3.6. Drug–excipient compatibility studies via Fourier transform infra-red spectroscopy (FTIR)

The Fourier transform infra-red spectra (FTIR) of all the proniosomes were obtained to ascertain if there would be any incompatibility or unpleasant interactions. The spectrum of the each of the carriers was compared to the pure drugs and drug loaded proniosomes. The lactose or dextrin loaded proniosomes containing either 5-FU or LEU showed prominent O-H stretch at wavenumber 3200–3300 cm $^{-1}$ while for the Neusilin based proniosomes, the spectrum showed absence of O-H stretches at this wavenumber (Fig. 5). Generally, there were no differences in the spectra of the carrier and the drug loaded formulations. Hydrogen bonding

b.

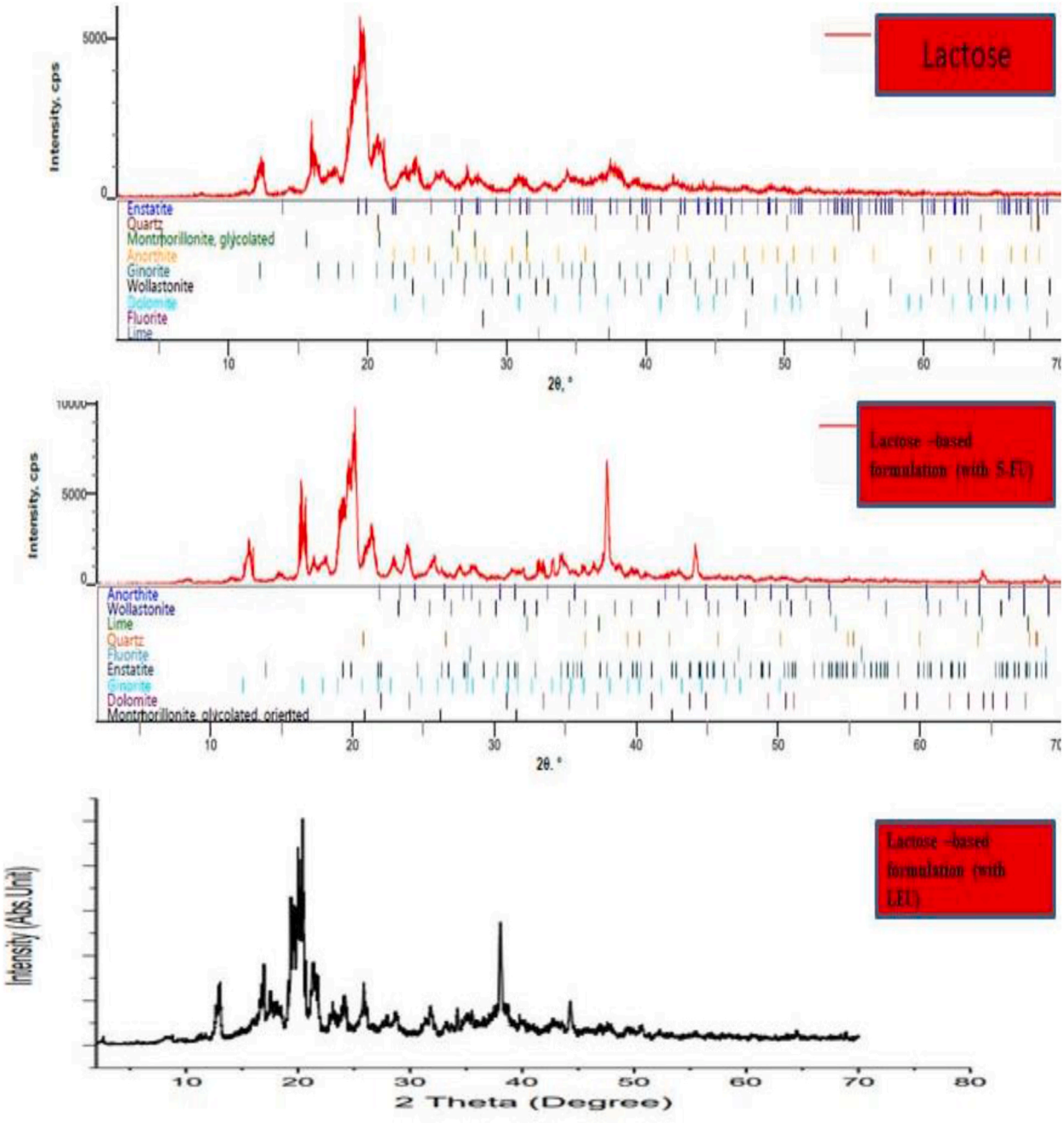


Fig. 4. (*continued*).

interactions has been reported with most niosomal formulations because of the presence of hydrogen acceptors/donors on the drugs and the noisome constituents e.g. cholesterol and surfactant [36–38]. The IR spectra of dextrin is depicted by peaks of O-H stretching vibrations at 3450 cm^{-1}, C-H stretching vibration at 2960 cm^{-1} and C-O stretching of the polysaccharide skeleton between 1000 and 1200 cm^{-1} [39]. Lactose is reported to show peaks at 3200-3600 cm^{-1}, 1650 cm^{-1}, and 1070 -1200 cm^{-1} signifying stretching vibration of the hydroxyl group, weak bending vibration of the hydroxyl group of water crystals and asymmetric stretching vibrations of C-O-C (in the glucose and galatose) respectively [40]. The IR spectra of lactose or the dextrin carrier (Fig. 5) is seen to possess OH groups which may have contributed to the hydrogen bonding interactions in the lactose or dextrin formulations. There was no

c.

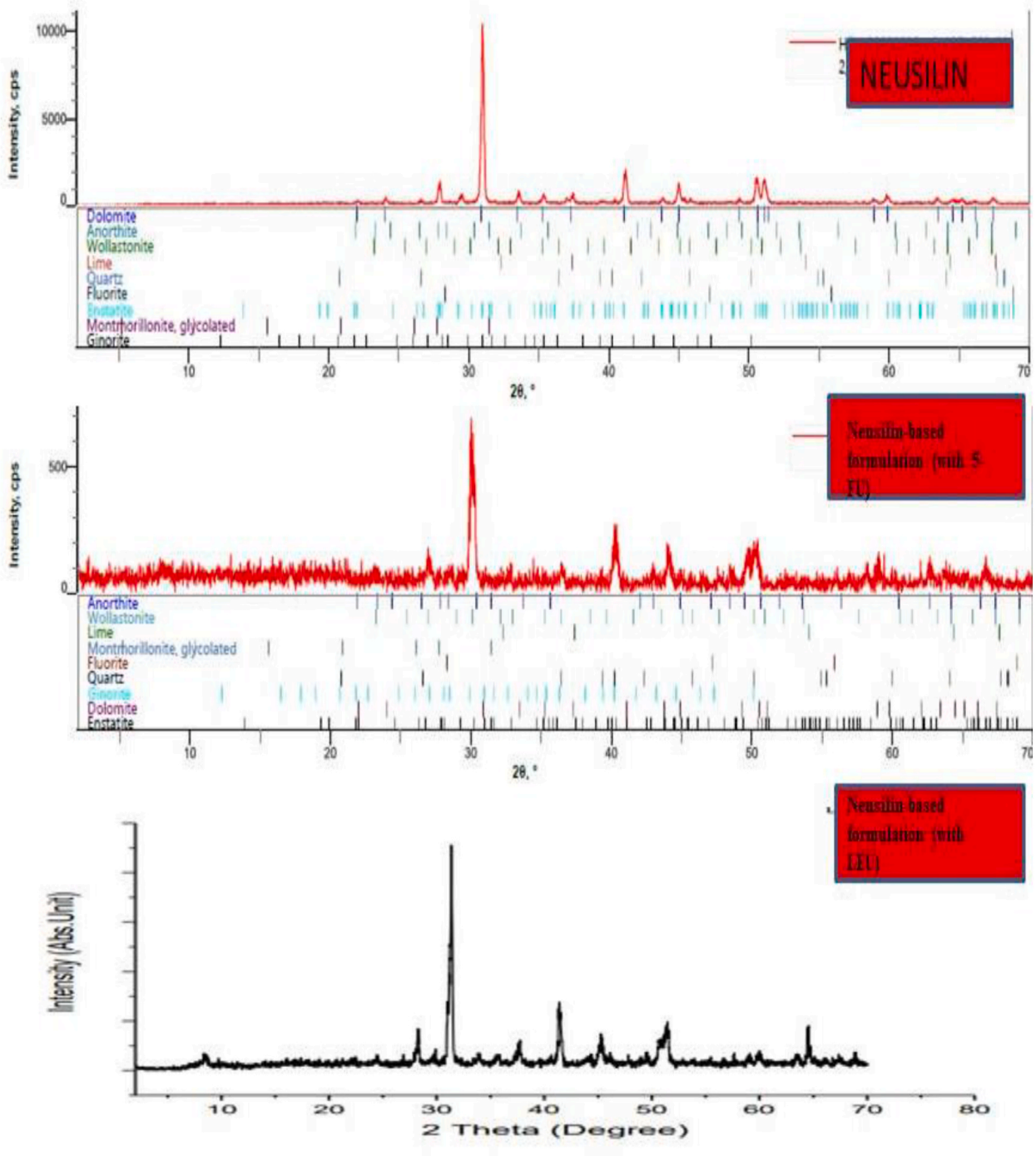


Fig. 4. (*continued*).

d.

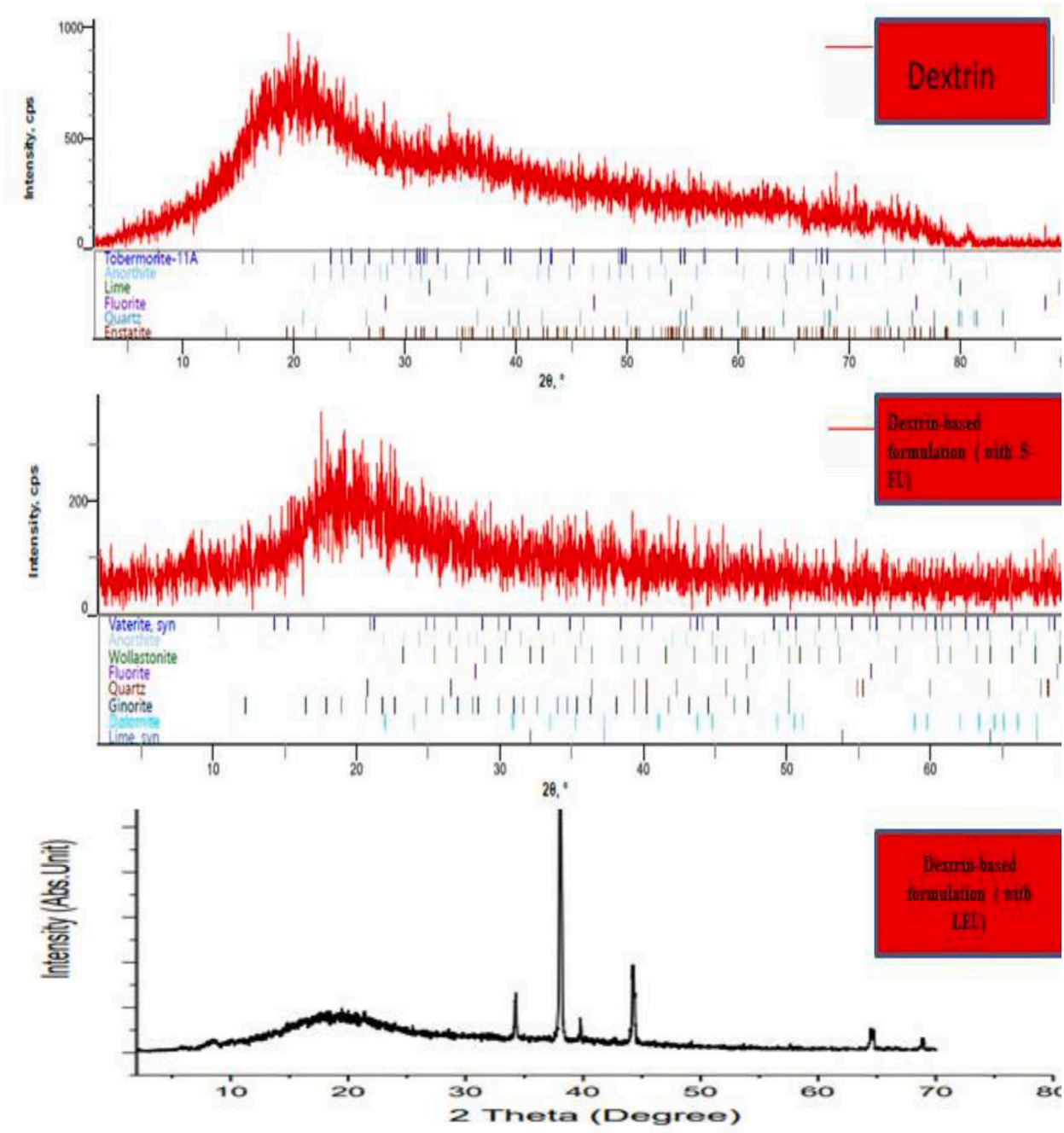


Fig. 4. (*continued*).

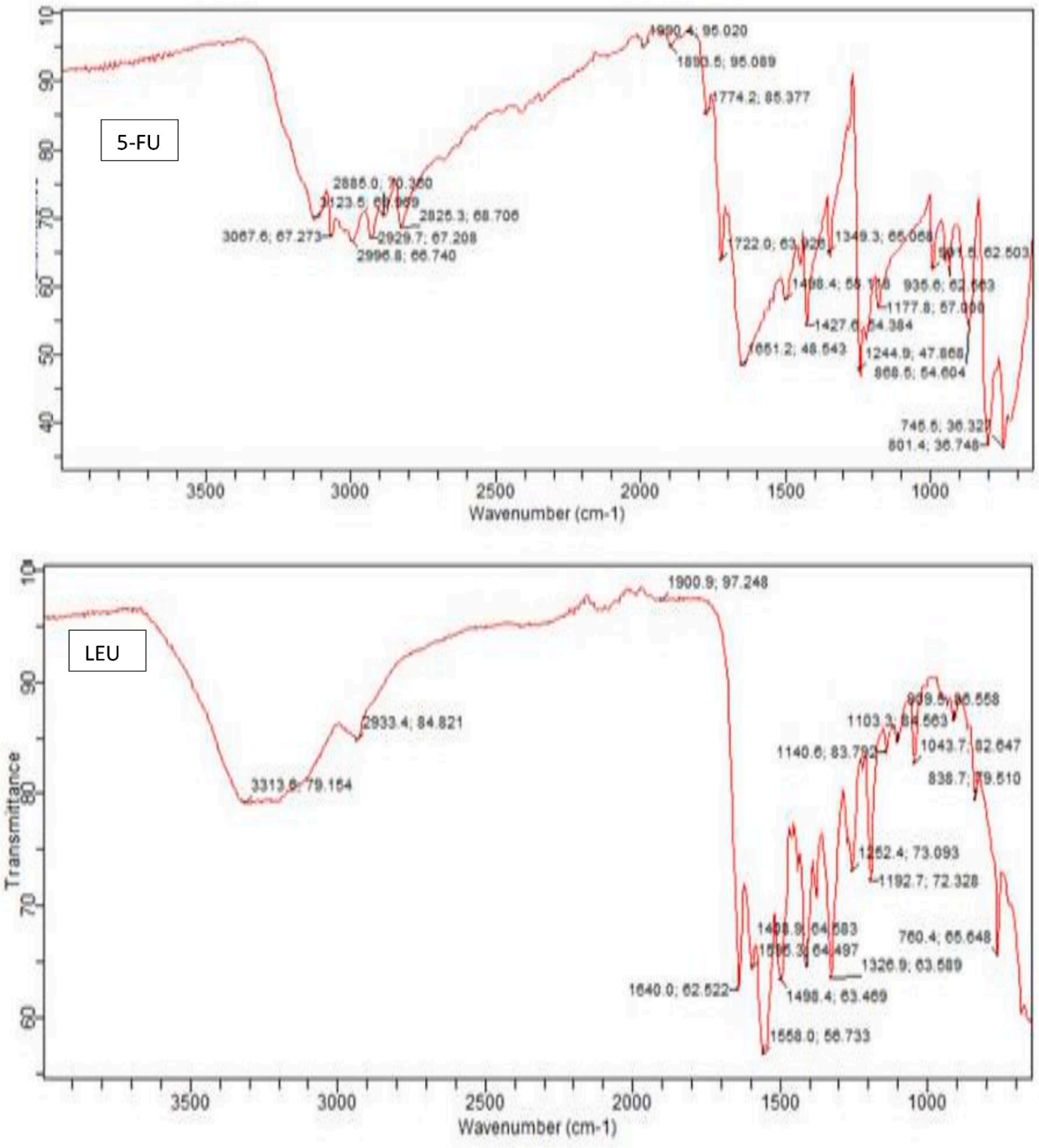


Fig. 5. FTIR spectra of pure drugs and proniosomal formulations
** 5-FU – 5- fluorouracil, LEU-Leucovorin, LAC- lactose, DEX – Dextrin, NEU – Neusilin, B & G – Dextrin –based formulations, H & C – Neusilin-based formulations, I & E – Lactose-based formulation

available site on the Neusilin structure for hydrogen bonding interactions to take place as was seen in the absence of vibrations above 3000 cm^{-1} wavenumber. The spectra showed no unpleasant interactions between the carriers, pure drugs and other excipients.

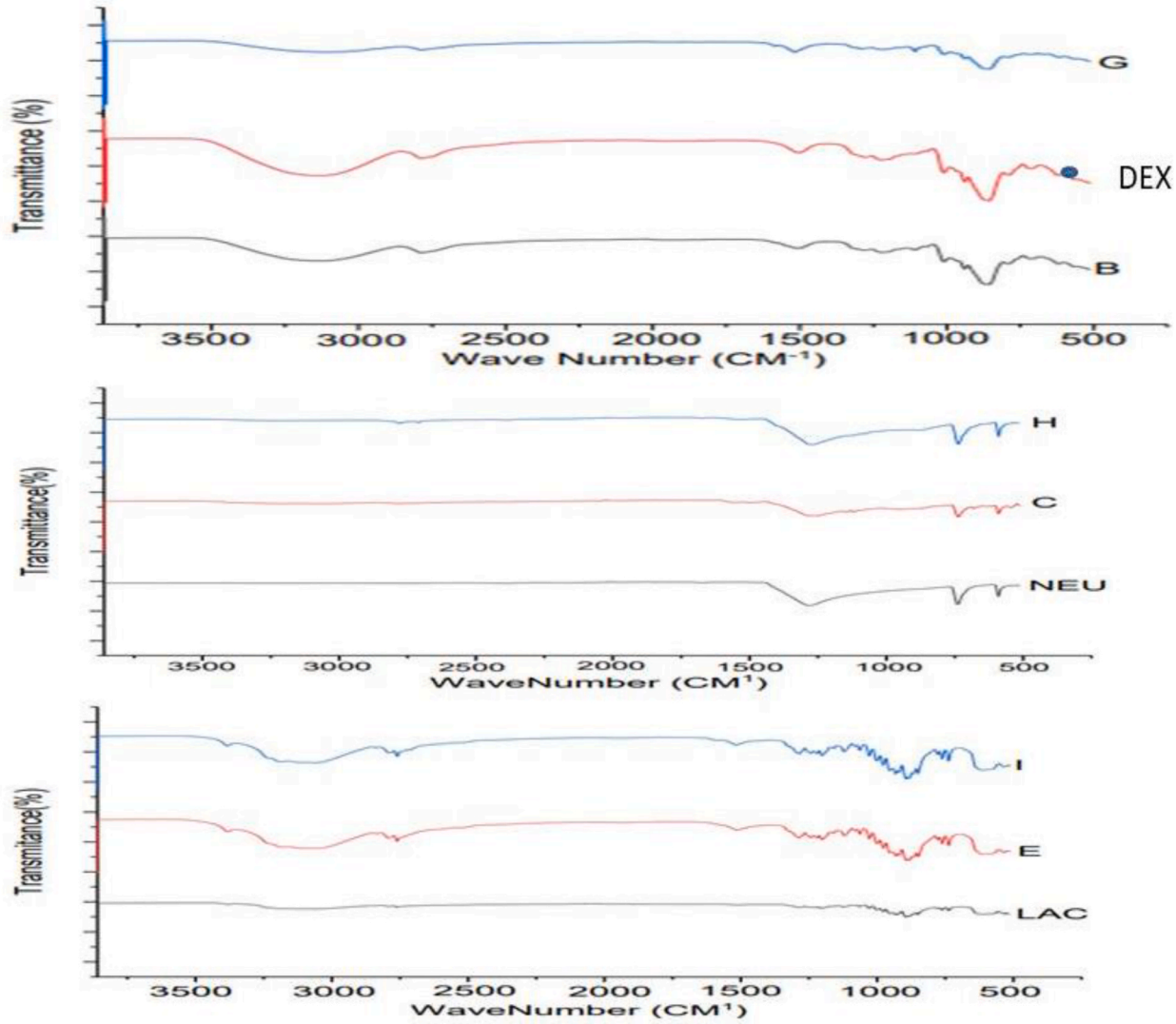


Fig. 5. (*continued*).

3.7. Drug release studies/dissolution studies for coated capsules and tablets

The coated capsules were prepared by dip coating in 10 % Eudragit S-100 solution; pre coated Eudragit FS30D capsules (Evonik Germany) were also used alongside to encapsulate the proniosomes. All the Eudragit S-100 coated formulations had 50 % increases in size as a result of the coating film deposited. The coating was to ensure the release of the drugs locally at the cancer site in the colon. The pH –dependent drug release from the encapsulated proniosomes was evaluated at pH values of 1.2 (for 2 h), 6.8 (for 3 h) and 7.4 simulating the stomach, small intestine and colon respectively. Release studies finding are presented in Fig. 6. There was negligible or no drug release (most were less than 10 %) seen in the SGF pH 1.2 in the first 2 h for all coated formulations. The Eudragit S-100 coated capsules showed a cumulative drug release between 33 and 56 % in the phosphate buffer pH 6.8, at the 5th h for most of the formulations. For the Eudragit FS30D capsules, cumulative drug release in the intestinal fluid (phosphate buffer pH 6.8) was between 8 and 15 % ($p < 0.05$) at the 5th h. The drugs started releasing rapidly at the 6th h when in contact with the colonic fluid (phosphate buffer pH 7.4) for all the formulations. The drug release at the 6th h was between 45.9 -77.17 % and 30–69 % for the Eudragit S-100 (ES) (P1 to P16) and Eudragit FS30D (EFS) capsules (FS1 to FS6) respectively. The pH- sensitive capsules continued to release the drug until maximum drug release was attained. Most of coated formulations containing the proniosomes had over 85 % cumulative drug release in the 10th h, however in the 24th h only a minimal increase in the drug released was observed. The maximum cumulative percentage drug release was between 71 – 100 % and 84–100 % for both ES capsules and EFS capsules containing proniosomes respectively. Eudragit S-100 and Eudragit FS 30D are poly (meth)acrylate based co-polymers with gastro resistant properties. They are insoluble at low pH, but they become increasingly soluble at higher pH. They have found usefulness in targeting drug moiety to the lower portion of the gastrointestinal tract (GIT) [41]. The colon is divided into 4 regions – ascending, transverse, descending and

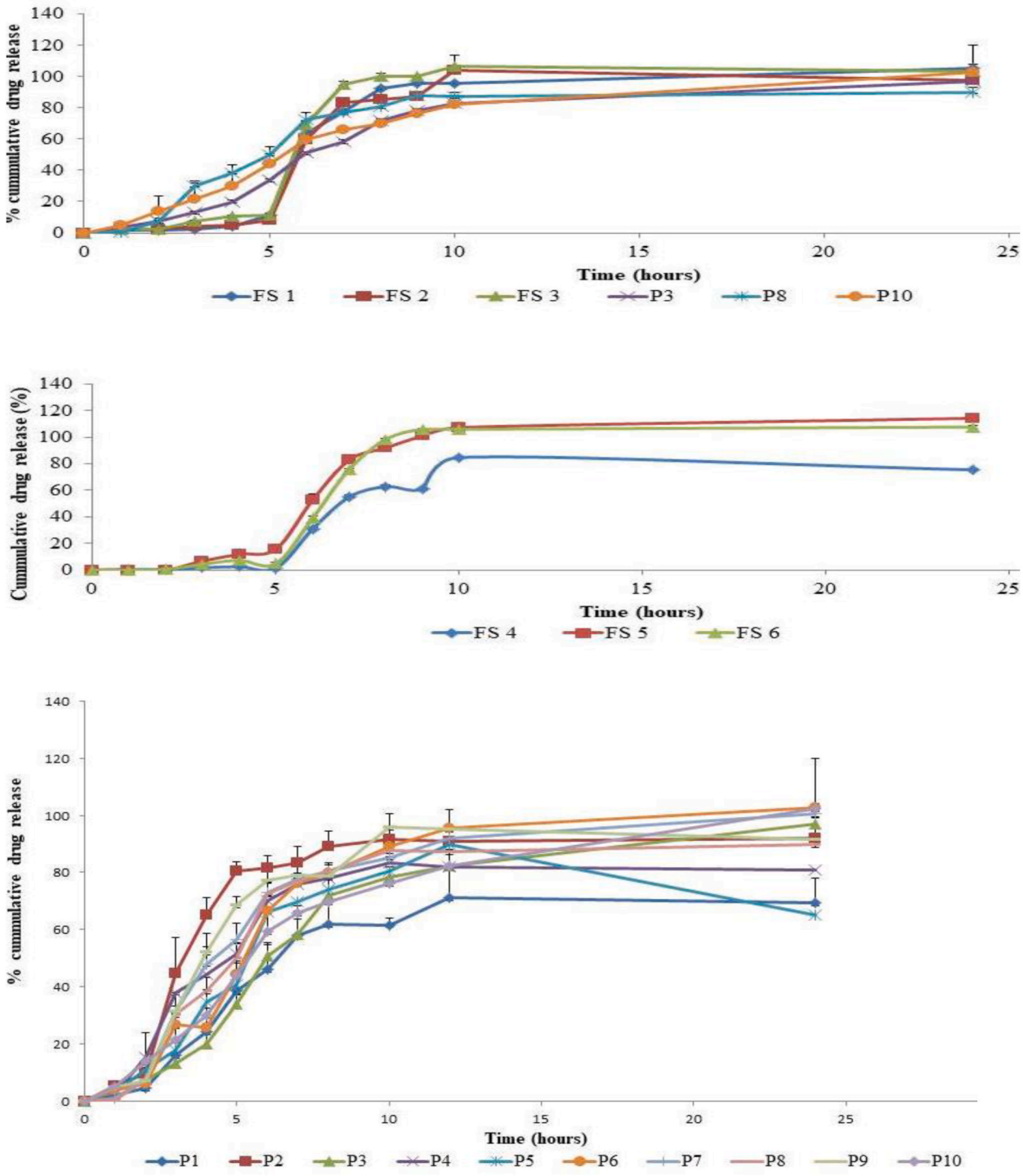


Fig. 6. *In vitro* drug release studies of colon-targeted (Eudragit S100 coated and Eudragit FS 30D) 5-FU/LEU-loaded proniosomes
***Time interval: 0–2 h – Acidic medium** pH **1.2; 3–5 h – alkaline medium** pH **6.8; 6–24 h – alkaline medium** pH **7.4**; **, P1, P10, FS1, FS4 – Neusilin-based proniosomes, P2, P9– lactose-based proniosomes, P3 – P8, FS2, FS3, FS5, FS6 – dextrin-based proniosomes; *P1-P10 depicts the Eudragit S100 coated capsules; FS1 – FS6 depicts Eudragit FS30 D capsules**.

rectosigmoid colon. A previous study by Bharucha et al. [42] provided interesting information about the movement of pH-sensitive capsules through the GIT by scintigraphy. The capsules were radiolabeled with technetium 99 m (99mTc) or indium 111 (111In) and the transit of the capsules was monitored. They observed that pH-sensitive capsules began to swell between pH 7.2–7.4, within the

distal ileum, and released its content into the ascending colon. Based on this information and the *in vitro* dissolution studies in this experiment, some of pH-sensitive capsules released most of its content in the colon environment at pH 7.4. It was observed that the ES capsules released more of its drug content in the intestinal pH (phosphate buffer pH 6.8), while most of the content of the EFS capsules was released at the colonic pH (phosphate buffer pH 7.4). This further buttresses the potential ability of the ES/EFS coated capsules to deliver drugs to the lower part of the small intestine and the colon. In the course of this study we must recall that our encapsulated proniosomes were granules which allowed for ease of hydration when the capsules were in contact with the colon fluid. On careful observation, the release of the capsule content was rapid in phosphate buffer pH 7.4 for all coated capsules. The proniosomes should hydrate fast in the colonic fluid *in vivo* and make the niosomal formulations ready for therapeutic action. We must also recall that most of the proniosomal formulations were less than 400 nm in particle size. Nano-scale particle sizes of the proniosomal formulations will impact the advantage of improved pharmacokinetics and bio-distribution of therapeutic agent due to high surface area to volume ratio. Reduced toxicity and increased intracellular penetration are also additional benefits of these formulations [43]. The drug loaded proniosomal formulation is expected to hydrate in the intestinal/colon fluid, pass through the biological barriers, saturate at disease site (colon) and elicit therapeutic actions. Targeted delivery to the colon is known to elicit desired local effect and to protect the drug from degradation in the stomach. 5-FU, an effective colon cancer drug is associated with short elimination half-life (16 min), parenteral presentation and increased catabolic breakdown in the liver, hence the reason for targeting it to the colon [44]. On the other hand, leucovorin is frequently used in conjunction with 5-FU to modify its therapeutic impact and toxicity in the treatment of colorectal tumors. In this study, the pH-sensitive polymers released the proniosomes passively in the colon; this nanoscale vesicular drug delivery will in turn promote good cellular uptake within the colon tissues, thereby reducing toxicity and improving therapeutic response. Although there were no significant differences in the release profiles of the various carriers, dextrin-based proniosomes (FS3, FS5, and P6) showed the highest drug release (>95 %). Dextrin-based proniosomes may provide additional benefits, as dextrin is broken down by bacterial polysaccharides in the colon [45]. In summary, ES capsules containing proniosomes released their drug content in the lower portion of the small intestine, while EFS capsules released over 80 % of their drug content in the colon environment. The EFS capsules (FS3 and FS5) proved to be more efficient for colon targeting, and it is our preferred formulation for treatment.

3.8. Release kinetics

Selected batches from the drug loaded proniosomes were fitted into the various mathematical release models to ascertain which release mechanism was prominent. Results are presented in Table 4. The R^2 statistically gives information of how best the formulations fit into the mathematical models. Most of the batches – FS2, FS3 and P3 fitted best in the Korsmeyer -Peppas model except FS1 which fitted best in the Hixson-Crowell model. When the rate of drug release is independent of the concentration of the drug, it is said to be zero order kinetics. For the first order kinetics, the rate of drug release is dependent on its concentration [46]. The Higuchi release model explains that the release of drugs from an insoluble matrix is dependent on the square root of time, anchored on Fickian diffusion [47]. Korsmeyer-Peppas model describes a system where the fractional release of drug is exponentially related to time while the Hixson-Crowell release model describes the release of drug from a system where there is an alteration in the diameter and the surface area of the tablets [48,49]. The kinetic study is usually employed to study release mechanisms. The value of "n" in the Korsmeyer-Peppas model indicates the mechanism of release. Values of $n \geq 0.45$ depict a Fickian diffusion mechanism; $0.45 < n < 0.89$ demonstrates non-Fickian transport; $n = 0.89$ illustrates Case II (relaxational) transport, and $n > 0.89$ also shows super case II transport [50]. A previous study on proniosomal gels of itraconazole reported the Higuchi model to be the best fit for their formulation [51]. Another study concluded that the Korsmeyer-Peppas model had the best fit for the formulation of transdermal captopril proniosomes [52]. In this study, most of the selected batches showed the best fit in the Korsmeyer-Peppas model. In summary,all formulations, except FS1, had $n > 0.89$, signifying that their mechanism of release was super case II transport, indicating that release occurred via the diffusion and relaxation of polymer chains.

3.9. In vitro cytotoxicity-real time cell assay (RTCA)

Cytotoxicity refers to the ability of a compound to kill cells. Cytotoxicity tests can be performed using various methods. The most common conventional method is the classic colorimetric test (MTT assay). However, the RTCA system has recently become more popular for cellular assays and experiments [53]. The RTCA system has the merits of reduced contamination risks, extra functionality, and the ability to record real-time data compared to the classic colorimetric test [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT). It is gradually replacing the conventional assay methods for cytotoxicity studies. RTCA works based on the principle of cell adhesion on the gold microelectrodes in the RTCA plate and records the impedance of the electric current flow as a cell index

Table 4
Release kinetics of the formulations.

Batches	Zero order (R^2)	First order R^2	Higuchi R^2	Hixson Crowell R^2	Korsmeyer-Peppas R^2	Korsmeyer-Peppas n
FS 1	0.582	0.711	0.628	0.748	0.639	0.697
FS 2	0.769	0.622	0.508	0.667	0.911	2.183
FS 3	0.777	0.606	0.523	0.660	0.868	1.676
P 3	0.937	0.828	0.677	0.867	0.989	1.758

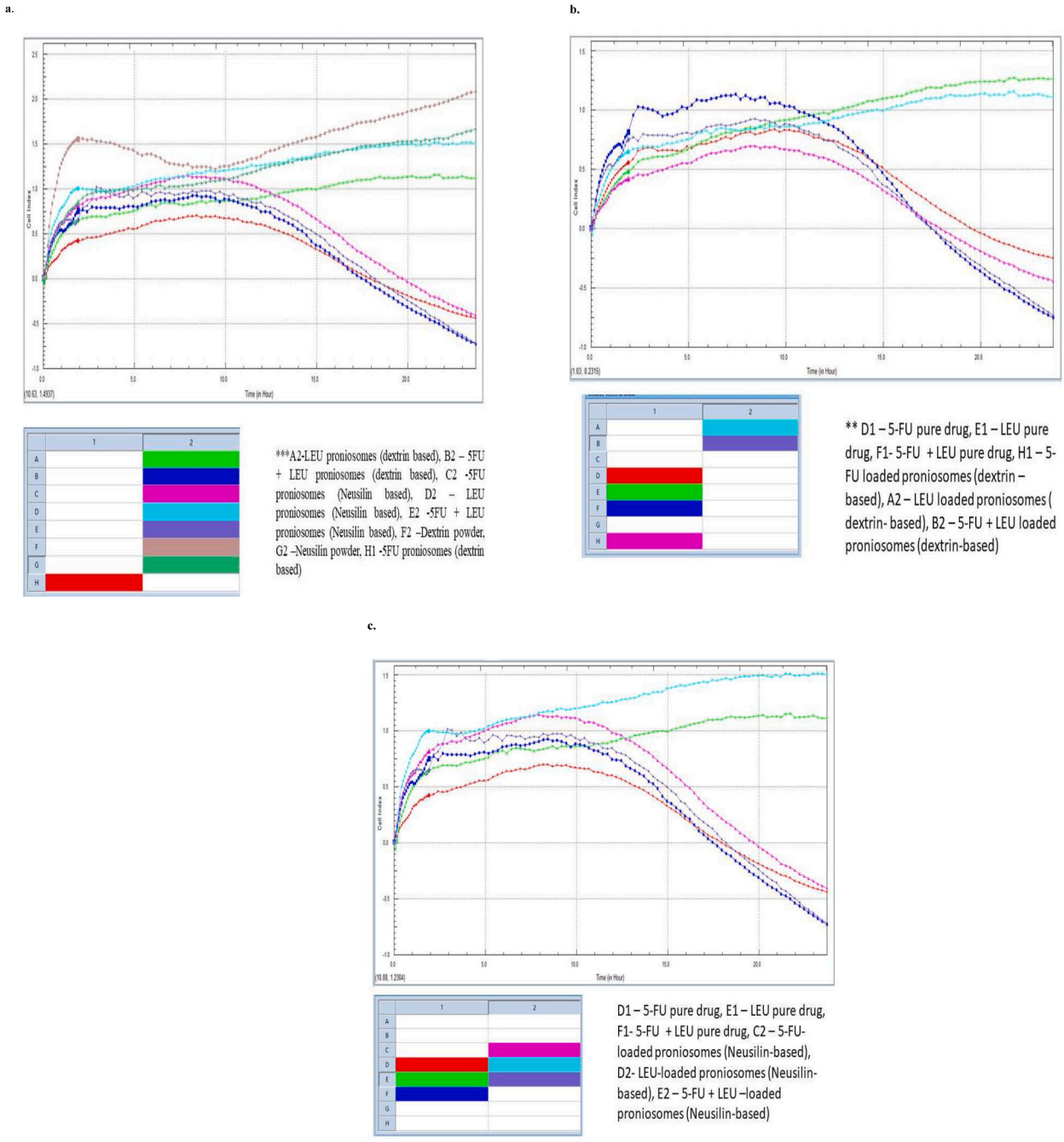


Fig. 7. *In vitro* cytotoxicity studies of 5-FU and LEU loaded prooniosomes against HCT-116 colon cell lines (comparing dextrin based, Neusilin based proniosomes and placebo).

(CI). When HCT-116 cells were seeded in an RTCA plate, cell adhesion and proliferation were observed to impede current flow, and the CI value increased [12,54]. In the presence of 5-FU or 5-FU plus LEU pure drug or proniosomes, decreasing CI over 24 h was observed due to the decreasing proliferation ratio (Fig. 7). The placebo (dextrin and Neusilin carriers) and LEU were observed to have increasing CI values, indicating increased cell adhesion and proliferation. This clearly shows that cytotoxicity was solely due to 5-FU and not the excipients used for formulation or LEU. Steady cell death (reduced proliferation), depicted by a constant reduction in cell index, and was observed with the 5-FU + LEU proniosomes within the 24 h test period. A cell index of 0 indicated no cell proliferation or adhesion [13]. The greater cytotoxicity observed in proniosomal formulations over the 24 h study period may be due to the gradual release of the drugs from the vesicles, as proniosomes have the advantage of controlled drug release over a prolonged period. Consequently, the reduced adverse effects of the drug will be a positive outcome for patients. In the 24 h study period, the dextrin-based proniosomes

(5-FU + LEU) (B2) showed a lower cell index (0.7 maximum) compared to Neusilin-based proniosomes (E2) with a maximum cell index of 1.0 maximum (Fig. 7). Dextrin is a water-soluble carrier; therefore, the dextrin-based formulation has the advantages of ease of hydration, quick onset of action, and higher bioavailability compared to the Neusilin-based formulation, which may have suffered incomplete desorption in the cells, reducing the bioavailability of drugs. It should be noted that the XRD studies (Section 3.5) showed dextrin-based proniosomes to be amorphous. Amorphous materials are associated with a higher solubility [35]. This may explain the better cytotoxicity observed with dextrin-based proniosomes. Dextrin and Neusilin powder had no cytotoxic effects because the cell index values continued to increase, resulting in increased cell proliferation or multiplication. The cell index profile improved when LEU was administered with 5-FU. Generally, lower cell index values were observed when the combination of 5-FU + LEU proniosomes or pure drug (B2, E2, F1) was administered compared to when only 5-FU proniosomes or pure drug (H1, C2, D1) were used. In summary, the controlled release pattern of 5-FU and LEU niosomes seen from real-time cell death records may result in reduced gastrointestinal and cardiac side effects associated with 5-FU.

4. Conclusion

5-Flourouracil and leucovorin proniosomes were successfully engineered and delivered to the colon using Eudragit S 100 (10 %)-coated capsules and/or Eudragit FS 30D®-coated capsules. Various *in vitro* evaluations and real-time cell assays (*in vitro* cytotoxicity tests) were performed for the formulations. Targeting the drug-loaded proniosomes to the colon allowed for more intimate interactions between the drugs and disease site. Powdered niosomes that facilitated easy handling while retaining all characteristics of the vesicular drug delivery system were produced. These formulations will improve the pharmacokinetics of the drugs (prolonging the drug effect), reduce adverse effects, and improve the quality of life of patients with colon cancer. Our study targeted these drugs to the disease site, where they are supposed to saturate and penetrate the tumor environment. Further studies are required to actively target colon cancer cells by taking advantage of overexpressed moieties on tumor cells via the application of ligand – nanocarrier conjugation chemistry.

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CRediT authorship contribution statement

Ugorji Onyinyechi Lydia: Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization. **Ikechukwu Virgilius Onyishi:** Writing – review & editing, Investigation. **Amarauche Chukwu:** Supervision, Resources, Formal analysis. **Anthony Amaechi Attama:** Writing – review & editing, Resources, Formal analysis.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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