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Development and evaluation of liquid crystal systems of combination of 5-fluorouracil and curcumin for cervical cancer cell line

[Desarrollo y evaluación de sistemas de cristal líquido de una combinación de 5-fluorouracilo y curcumina en una línea celular de cáncer cervical]

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Abstract

Context: Liquid crystalline gel, self-assembled vesicular systems covered with nonionic surfactants, are promising for use in drug delivery.

Aims: To develop and evaluate the liquid crystal (LC) systems of a combination of 5-fluorouracil and curcumin for a cervical cancer cell line.

Methods: The LC was formulated using water, surfactant, and glycerin. Optimization was carried out by using different surfactants. The formulations were chosen for various studies, such as pH determination, environmental scanning electron microscopy (eSEM), Fourier transform infrared spectroscopy (FTIR) and dissolution study. The synergism of the combination of 5-FU and curcumin on cervical cancer HeLa cell line was then analyzed using CompuSyn software.

Results: The formulations with 60% surfactant, the average particle size was in the ranges from 15.68 – 26.35 nm whereas with 40% surfactant, were 11.27 - 21.35 nm. The percentage of dissolution of 5-FU at pH 7 (PBS) A2: 40.23 > B2: 47.39 > X2: 50.36 > Y2: 56.36 compared to at pH 4 (vaginal simulated fluid, VSF) were A2: 36.23 > B2: 43.64 > X2: 45.67 > Y2: 49.01. The percentage of dissolution of curcumin of different formulations were at pH 7 (PBS) A2: 44.85 > B2: 51.36 > X2: 58.61 > Y2: 64.38 compared to at pH 4 (VSF) were A2: 41.03 > B2: 49.37 > X2: 57.85 > Y2: 68.75.

Conclusions: The combination of the drugs showed that there was a synergistic effect when it was being administered together. The combination of drugs in LC system had a sustained release as well as it was effective against cervical cancer HeLa cell line.

Keywords: cervical cancer; curcumin; 5-fluorouracil; HeLa cell line; liquid crystal.

Resumen

Contexto: El gel cristalino líquido, los sistemas vesiculares autoensamblados cubiertos con tensioactivos no iónicos, son prometedores para su uso en la administración de fármacos.

Objetivos: Desarrollar y evaluar los sistemas de cristal líquido (LC) de una combinación de 5-fluorouracilo y curcumina para una línea celular de cáncer cervical.

Métodos: El LC se formuló usando agua, tensioactivo y glicerina. La optimización se llevó a cabo utilizando diferentes tensioactivos. Las formulaciones se eligieron para diversos estudios, como la determinación del pH, la microscopía electrónica de barrido ambiental (eSEM), la espectroscopía infrarroja por transformada de Fourier (FTIR) y el estudio de disolución. Luego se analizó la sinergia de la combinación de 5-FU y curcumina en la línea celular HeLa de cáncer de cuello uterino utilizando el software CompuSyn.

Resultados: Para las formulaciones con 60% de tensioactivo, el tamaño de partícula promedio estuvo en el rango de 15,68 – 26,35 nm mientras que con 40% de tensioactivo, fueron 11,27 – 21,35 nm. El porcentaje de disolución de 5-FU a pH 7 (PBS) A2: 40,23 > B2: 47,39 > X2: 50,36 > Y2: 56,36 en comparación con pH 4 (fluido simulado vaginal, VSF) fueron A2: 36,23 > B2: 43,64 > X2: 45,67 > Y2: 49,01. El porcentaje de disolución de curcumina de diferentes formulaciones fue a pH 7 (PBS) A2: 44,85 > B2: 51,36 > X2: 58,61 > Y2: 64,38 en comparación con pH 4 (VSF) fueron A2: 41,03 > B2: 49,37 > X2: 57,85 > Y2: 68,75.

Conclusiones: La combinación de los compuestos mostró que había un efecto sinérgico cuando se administraba en conjunto. La combinación de fármacos en el sistema LC tuvo una liberación sostenida y fue eficaz contra la línea celular HeLa del cáncer de cuello uterino.

Palabras Clave: cáncer de cuello uterino; cristal líquido; curcumina; 5-fluorouracilo; línea celular HeLa.

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INTRODUCTION

Cervical cancer is defined as an abnormal cell growth affecting the cells lining the cervix (Cohen et al., 2019). Both the National Institute of Health and the National Cancer Registry, Malaysia, stated that cervical cancer is the third leading cancer among women in Malaysia and worldwide in the years of 2008 and 2006 (Ministry of Health Malaysia, 2006). There are two types of cells lining the cervix, squamous cells and glandular cells and most cervical cancers are usually squamous cell carcinoma (Cohen et al., 2019). The main cause of cervical cancer is the human papilloma virus, which can be detected using the Pap smear test (Cohen et al., 2019).

Liquid crystal is a substance that possesses the properties of both liquid and solid where it may flow like water but the molecular structure of liquid crystal represents a crystal (Rajabalaya et al., 2017). Liquid crystal system is suitable to be used locally since the system has a bio-adhesion and penetration enhancement property thus the absorption of drugs is faster (Bei et al., 2009; Rajabalaya et al., 2017). Due to the large internal surface area and honeycombed structure of the liquid crystal system, it allows high drug loading and enables incorporation of drugs with different physicochemical properties, hydrophilic, lipophilic and amphiphilic substances other than having a property of a sustained release drug delivery system (Rajabalaya et al., 2017). Curcumin or diferuloylmethane is an active ingredient extracted from the rhizomes of *Curcuma longa* L. (family *Zingiberaceae*) or also called as turmeric (Tuyaerts et al., 2019). Traditionally, it has been used as a spice in cooking and also used to treat illnesses such as infection of the bile duct and gall bladder as well as other inflammatory diseases (Tamvakopoulos et al., 2007). Curcumin has also been shown to have anti-oxidant, anti-proliferative and anti-inflammatory properties (Tamvakopoulos et al., 2007).

5-Fluorouracil (5-FU) is a widely known anti-metabolite that has been used in the treatment of cancer for many years. 5-FU competes with natural substrates for thymidylate synthetase; thus, it is incorporated into RNA or DNA as false substrate. In the treatment of cervical cancer, 5-FU is usually given together with cisplatin or paclitaxel. The combination of curcumin and 5-FU has been studied widely. Some studies showed that there is a synergistic effect between curcumin and 5-FU (Du et al., 2006; Ahn et al., 2010; Liu et al., 2017). The objectives of this research were to determine the suitable solvents and adjuvants for liquid crystal systems and formulation, to study the physicochemical properties of the liquid crystal systems and the formulation, to study the dissolution of the drug and to the analyze the efficacy of the formulation on a cervical cancer cell line.

MATERIAL AND METHODS

Materials

Curcumin was purchased from Pi Chemicals Ltd. (Shanghai, China). The surfactant Polysorbate 80 (Tween 80) was acquired from EMD Millipore Corporation, Billerica, MA, USA. Glycerol, phosphate buffer saline (PBS) tablets, Dulbecco's modified Eagle's medium (DMEM), and bovine serum albumin were acquired from Sigma-Aldrich, St Louis, MO, USA. Caprylocaproyl macrogol-8-glyceride (Labrasol®) was kindly provided from Gattefossé (St Priest, France) and polyoxyl 35 castor oil (Cremophor® EL) from BASF (Ludwigshafen, Germany). All other chemicals used were of analytical grade.

Curcumin and 5-FU combination liquid crystal preparation and optimization

Formulations of the liquid crystal were prepared based on studies carried out by Rajabalaya et al. (2017) and Das et al. (2018). Tween 80 was used as the surfactant while glycerin and water were used as the aqueous phase. Curcumin, which is lipophilic in nature, was mixed with Tween 80 and melted in water bath with constant sonication for 15 minutes. On the other hand, 5-FU, which is hydrophilic, was dissolved in water first, before adding to glycerin. Both parts were then poured into a universal bottle and mixed thoroughly using

a vortex mixer (Rajabalaya et al., 2016). The formulation was optimized using three different surfactants, Tween 80, Labrasol and Cremophor with four different concentrations of surfactant 20, 40, 60 and 80% and using three different concentrations of drug, 0.1, 0.3 and 0.5%.The formulations that met the optimal physiochemical properties were used for further research.

Physiochemical investigation

Organoleptic properties

Organoleptic properties such as odor and color were determined by using the LC samples.

Homogeneity test

A 0.5 gm of a sample was taken and pressed between the index finger and thumb to check on the consistency of the formulation.

pH determination

Accurately weighed amount (0.5 g) of the sample formulation was dissolved in 15 mL distilled water (pH 7.0) and the pH was measured using a calibrated pH meter, Mettler Toledo, USA (Table 1).

Encapsulation efficiency

The LC formulations were centrifuged at 10,000 rpm at a temperature of 5°C for 60 min in two cycles using a cooling centrifuge (Eppendorf® Model 5810R, Eppendorf, Hamburg, Germany) to isolate the drug-carrying vesicles from the unentrapped drug. The supernatant was then removed and lysed with methanol. The sediment was filtered through a 0.45 µm nylon disk filter and the free drug amount in the supernatant was measured using High-Performance Liquid Chromatography (1200 HPLC series, Agilent Technologies, Santa Clara, CA, USA) (Musa et al., 2017). The percentage encapsulation efficiencies were determined based on the following formula [1] (David et al., 2013) and are presented in Table 1.

$$\% \text{ Encapsulation efficiency} = \frac{\text{Total drug content - drug content in supernatant}}{\text{Total drug content}} \times 100 \quad [1]$$

Particle size determination

An aliquot of 0.1 g of LC formulation was diluted with 5 mL double distilled water and sonicated for 30 seconds in an ice bath. Particle size of the sample was analyzed at a scattering angle of 173° at a temperature of 250°C (Rajabalaya et al., 2016). One mL of solution was injected into a zetasizer cuvette and scanned in Malvern Zetasizer Nano ZS (Malvern Instruments Limited, United Kingdom) (Table 1).

Environmental Scanning Electron Microscopy (ESEM)

A LC gel weighing 0.1 g was placed on a holding disk and a drop of distilled water was added. The disk was placed in a chamber inside an Environmental Scanning Electron Microscope (eSEM, Quanta 450 FEG; FEI, Hillsboro, OR, Fei Company, USA). Water within the sample was allowed to evaporate before examination. The sample formulation was placed in the sample holder and scanned (Rajabalaya et al., 2016).

Fourier Transform Infrared Spectroscopy (FTIR)

The IR spectra of the drugs, 5-FU crystal and powdered curcumin were measured individually and as combination by mixing both ingredients. The drugs combination was made into powder form before adding KBr. The thin film, formed by compressing the drug powder and KBr disc, was analyzed using Shimadzu FTIR-8400S FTIR spectrometer at the wavelength of 500 – 4000 cm^{-1} (Rajabalaya et al., 2013).

Dissolution

Dissolution study was carried out to analyze the *in vitro* drug release profile of the drugs. Dissolution study was carried out in modified USP dissolution apparatus II (Weng and Parrott, 1983) for release studies of gels using two different simulated conditions, in vaginal simulated fluid (VSF) and PBS. VSF was prepared with the method suggested by Owen and Fatz (1999) The weighed amount (5 g) of gel was placed in 900 mL of vehicle (Weng and Parrott, 1983), VSF/PBS at 37 ± 0.5°C with paddle speed set to 50 rpm. The dissolution medium (900 mL) for gels and combination gels was

0.5% (m/v) solution of sodium lauryl sulphate in water (Owen and Katz, 1999). The 200 µL sample from the dissolution medium was taken every hour for 8 hours and replaced with their respective vehicle. The samples taken were made up to 10 mL and the absorbance were determined using dual beam UV-Visible spectrophotometer, PerkinElmer, USA, at wavelengths of 250 nm and 421 nm for 5-FU and curcumin, respectively.

Drug efficacy study

Cervical cancer (HeLa) cell line was used in this study. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS) and 1% penicillin in an incubator (RS Biotech Laboratory Equipment, UK) supplied with 5% carbon dioxide. The cells were then plated onto 96-well plate and treated with blank liquid crystal, curcumin alone, 5-FU alone, combination of both drugs or molecular grade dimethyl sulfoxide (DMSO) as control. MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide), a yellow tetrazole, was then added to the plate. DMSO of analytical grade was then used to break down MTT into purple formazan, which stained the living cells. The viability of the cells was measured using Opsys MRTM Microplate Reader (Dynex Technologies Inc., USA).

Statistical analysis

Statistical analyses of data were undertaken using SPSS v8.0 (IBM, Armonk, NY, USA). Analysis of variance and the paired t-test were applied. $p<0.05$ was considered significant. Values are expressed the mean ± standard deviation.

RESULTS

Curcumin and 5-FU combination liquid crystal preparation and optimization

The results of the formulations prepared with Tween 80, Labrasol and Cremophor for percentage of cytotoxicity of different surfactants on HeLa cells have been tabulated in Table 2. In terms of toxicity, both Tween 80 and Labrasol did not elicit toxic effect on the cells unlike Cremophor at concentrations above 3 µg/mL. Furthermore, the formulations with Labrasol did not follow the desired criteria for vaginal drug delivery. Thus, based on these results, Tween 80 was selected and used in further experiments. Table 1 shows the results for the various physiochemical parameters and the formulation composition of the formulations.

Physiochemical investigation

All liquid crystal formulations prepared with Polyoxyethylene (20) sorbitan monooleate, also known as Tween 80, were light yellow in color, oily smell like odor and homogenous in nature. The pH measured for formulations ranged from 5.52 to 5.94 (Table 1).

Encapsulation efficiency

The encapsulation efficiency (EE) of 5-FU and curcumin are presented in Table 1. The percentage of encapsulation efficiency of the 5-FU ranged from 70.58 – 87.13%, where X2 exhibited the highest and curcumin oscillated between 74.10 – 91.20%, where the highest was for B2.

Particle size

Particles in formulations above 25:75 (5-FU:curcumin) were higher compared to other formulations.

For formulations with 60% surfactant, the average particle size was in the range of 15.68 – 26.35 nm while for formulations with 40% surfactant was between 11.27- 21.35 nm. The polydispersity index (PDI) values for the LC formulations were between 0.1 - 0.4 indicating that most of the vesicles ranged from homogenous to heterogeneous in particle size (Jukanti et al., 2011). In this study, four formulations with 50:50 ratio of drugs were shown to have PDI values between 0.1 - 0.2. The zeta potential ranges between -18 to -28 mV for all the formulations.

Table 1. Formulation composition and physiochemical properties of liquid crystal formulation.

Formulation Code	Drug: Tween 80 (%)	5-FU: Curcumin	pH	Encapsulation efficiency (%) ± SD		Vesicle size (nm) ± SD	Polydispersity index	Zeta potential (mV)
				5- FU	Curcumin			
A1	0.3:40	25:75	5.59	72.05 ± 2.84*	74.10 ± 2.09**	14.67 ± 0.58*	0.132 ± 0.05	-22.3 ± 1.02
A2	0.3:40	50:50	5.61	84.23 ± 3.64*	86.93 ± 1.37*	21.35 ± .056*	0.121 ± 0.02	- 26.2 ± 2.47
A3	0.3:40	75:25	5.54	79.10 ± 2.71*	80.39 ± 1.33*	20.24 ± 1.38*	0.424 ± 0.14	- 19.2 ± 1.14
B1	0.5:40	25:75	5.52	74.12 ± 1.25*	76.94 ± 2.45*	11.27 ± 1.27*	0.402 ± 0.15	- 18.1 ± 1.04
B2	0.5:40	50:50	5.53	86.35 ± 1.67*	91.20 ± 1.84*	16.35 ± 2.30*	0.236 ± 0.04	- 23.2 ± 1.74
B3	0.5:40	75:25	5.50	80.23 ± 1.57*	84.45 ± 2.93*	20.23 ± 2.64*	0.415 ± 0.11	- 17.4 ± 1.05
X1	0.3:40	25:75	5.85	70.58 ± 2.69*	74.45 ± 1.74**	11.36 ± 0.67*	0.187 ± 0.09	-23.5 ± 1.02
X2	0.3:60	50:50	5.67	87.13 ± 2.54*	89.48 ± 3.91*	22.67 ± 1.05	0.130 ± 0.07	- 28.3 ± 1.07
X3	0.3:60	75:25	5.68	76.69 ± 1.10*	79.83 ± 2.98*	25.67 ± 1.89*	0.408 ± 0.10	- 20.5 ± 1.14
Y1	0.5:60	25:75	5.52	73.15 ± 2.34*	75.74 ± 1.03**	15.68 ± 0.23**	0.428 ± 0.12	- 23.5 ± 1.16
Y2	0.5:60	50:50	5.94	83.27 ± 2.02*	86.05 ± 1.66*	21.67 ± 1.89*	0.204 ± 0.04	- 28.8 ± 1.83
Y3	0.5:60	75:25	5.84	79.07 ± 2.56**	81.74 ± 1.83*	26.35 ± 0.75*	0.406 ± 0.10	- 24.8 ± 1.32

Ratio for water: glycerin for all the formulations were 7:1; All formulations are Light-yellow, oily smell and homogenous in nature. The results are presented as mean ± SD. *p <0.01 and **p<0.001 indicate significant differences with respect to baseline valor of their respective group or among groups as shows the column at right. The one-way analysis of variance test followed by Bonferroni's multiple comparison between the formulations.

Table 2. The percentage of cytotoxicity of different surfactants on HeLa cells.

Concentration (µg/mL)	Percentage of cytotoxicity		
	Tween 80	Labrasol	Cremophor
0.75	0.02 ± 0.001*	0.04 ± 0.003*	0.5 ± 0.001*
1.50	0.04 ± 0.002*	0.02 ± 0.002*	0.6 ± 0.002*
3.00	0.04 ± 0.001**	0.06 ± 0.001*	0.9 ± 0.001**
6.25	0.04 ± 0.002*	0.08 ± 0.002*	5.7 ± 0.4*
12.50	0.04 ± 0.001*	0.09 ± 0.002**	8.2 ± 0.06*
25.00	0.04 ± 0.002*	1.6 ± 0.3*	30.8 ± 1.05*
50.00	0.04 ± 0.003*	1.8 ± 0.2*	45.5 ± 1.3*
100.00	0.04 ± 0.001*	2.4 ± 0.3*	82.5 ± 1.8*

The results are presented as mean ± SD. *p <0.01 and **p<0.001 indicate significant differences with respect to baseline valor of their respective surfactant. The one-way analysis of variance test followed by Bonferroni's multiple comparison between the formulations.

Environmental Scanning Electron Microscopy (eSEM)

Based on the eSEM results, the majority of the particles were in the size of 5 µm. The particle sizes from eSEM and from zetasizer were different due to the nature of the method used. It was observed that there were spherical structure formations of micelles in each formulation. The results have been shown in the Fig. 1A-D. Fig1C and 1D showed dark spots in the vesicles.

Fourier transforms infrared spectroscopy (FTIR)

FTIR spectra of the drug with combinations of Tween 80 were obtained.

Both 5-FU and curcumin showed its characteristic peaks of bands at 3425.69 cm^{-1} – 3028.31 cm^{-1} and 1500.67 cm^{-1}. Also, an additional band at 1431.23 was observed for curcumin. The bands at 1658.84 cm^{-1} and 1222.91 cm^{-1} are attributed by to (C=O) and δ (N-H) of 5-FU, respectively (Wu et al., 2015) (Fig. 2). (Derenne et al., 2013). The band at 1431.23 cm^{-1} is attributed to C=C aromatic while the band at 1500.67 cm^{-1} is for C=C olefinic of curcumin (Rajabalaya et al., 2015). The bands at 1658.84 cm^{-1} and 1222.91 cm^{-1} are attributed to C=O and δ (N-H) of 5-FU, respectively (Wu et al., 2015) (Fig. 2).

Dissolution

The graphs in the Fig. 3A-B, depicted that the percentage of drug dissolution of 5-FU, was higher (A2: 40.23%) at PBS pH compared to VSF pH (A2: 36.23%). In the Fig. 4A-B, the highest percentages of drug dissolution of 5-FU were at pH 7.4 (PBS: 56.36%) and pH 4 (VSF: 49.01%), respectively. The percentage of dissolution of 5-FU at pH 7(PBS) A2: 40.23 > B2: 47.39 > X2: 50.36 > Y2: 56.36 compared to pH 4 (VSF) A2: 36.23 > B2: 43.64 > X2: 45.67 > Y2: 49.01. The percentages of dissolution of curcumin of different formulations were at pH 7 (PBS) A2: 44.85 > B2: 51.36 > X2: 58.61 > Y2: 64.38 compared to pH 4 (VSF) A2: 41.03 > B2: 49.37 > X2: 57.85 > Y2: 68.75 (Fig. 3C-D).

Drug efficacy study

Determination of half maximal inhibitory concentration (IC_{50}) of the combination of curcumin and 5-FU on HeLa cells was determined. From the Fig. 4, it could be observed that the IC_{50} for curcumin and drug combination was 22.8 and 40.0 µg/mL respectively, while the IC_{50} for 5-FU was higher, thus it is not stated in Fig. 4.

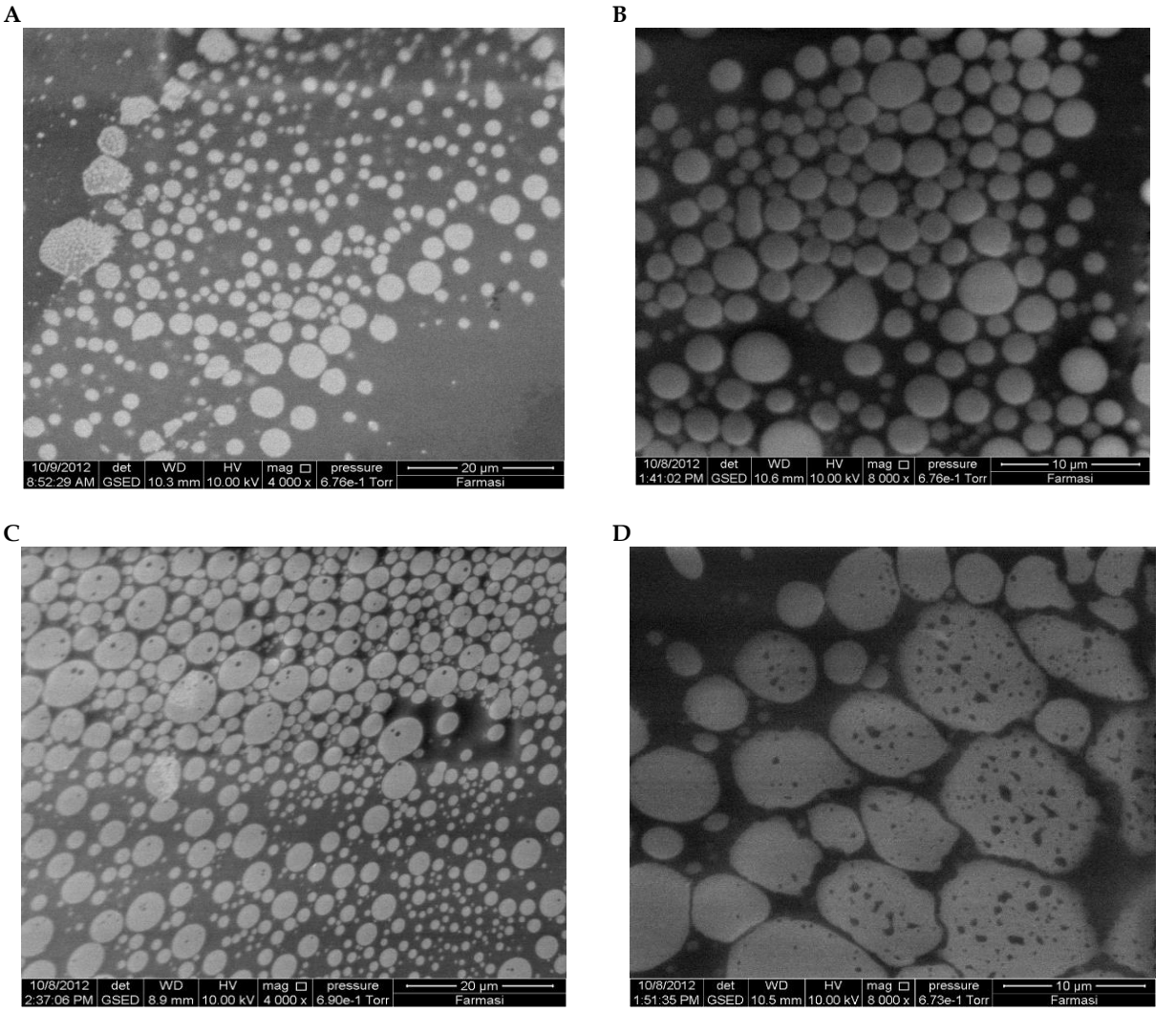


Figure 1. ESEM figures of **(A)** blank liquid crystal, **(B)** formulation A2, **(C)** formulation X2 and **(D)** formulation Y2.

DISCUSSION

Physiochemical properties

The formulations prepared need to follow the desired criteria where it needs to have gel-like appearance and do not contribute to any toxic effects to the cells. LC prepared with both Labrasol and Cremophor do not follow the desired criteria, namely pH and toxicity profile. So, they were not used in further studies. This finding was supported by previous studies, where it was reported that Cremophor EL increased the toxicity and lead to hypersensitivity reactions in certain individuals (Zhao et al., 2007). Therefore, Tween 80 with appropriate properties for vaginal drug delivery was selected and used for further studies.

The LC formulations contained inadequate amounts of aqueous phase; therefore, the pH values were determined for quality control and to evaluate the compatibility with vaginal pH. All the LC gel formulations were within the range of normal vaginal pH (Lucero et al., 1994). The ob-

tained pHs were suitable to be used in vaginal application (Musa et al., 2017).

LC prepared with 40 and 60% of Tween 80 were chosen as final formulations for subsequent studies accredited to their physical properties. In addition, Tween 80 has also been reported to have been used in the preparation of nanoparticles and nanoemulsions (de Mattos et al., 2015; Bonferoni et al. 2019) ascertaining the utility of the surfactant to be safe in various formulations.

Encapsulation efficiency

EE controls the important positive relationship with oil to the concentrations of surfactants and water. The encapsulation of 5-FU and curcumin is primarily due to its capability to liquify in the molten oil phase and its partition between the hydrophilic phase and surfactant (Das et al., 2018). All the LC formulations showing higher values of the EE, may be due to lesser bilayer permeability as well as higher lipophilic bilayer, leading to effective interpolation of the lipophilic drugs inside the core of the hydrophobic bilayers (El-Samaligy et al., 2006). However, the EE values do not significantly increase with increasing amounts of surfactant or drug contents. The higher amount of drugs and Tween 80 may cause the hydrophilic phase viscosity to increase, which then leads to the controlled drug release from the vesicles (David et al. 2018). It is postulated that lower quantity of aqueous phase leads to higher viscosity of LC formulation vesicles with bilayer formation ushering in accommodation of higher amount of drug in the surfactant hydrophobic chains (El-Samaligy et al., 2006; Rajabalaya et al., 2016). This would aid in the formation of suitable viscosity and phase of LC gels phase, subsequently supporting the delivery of the drug in a controlled release manner.

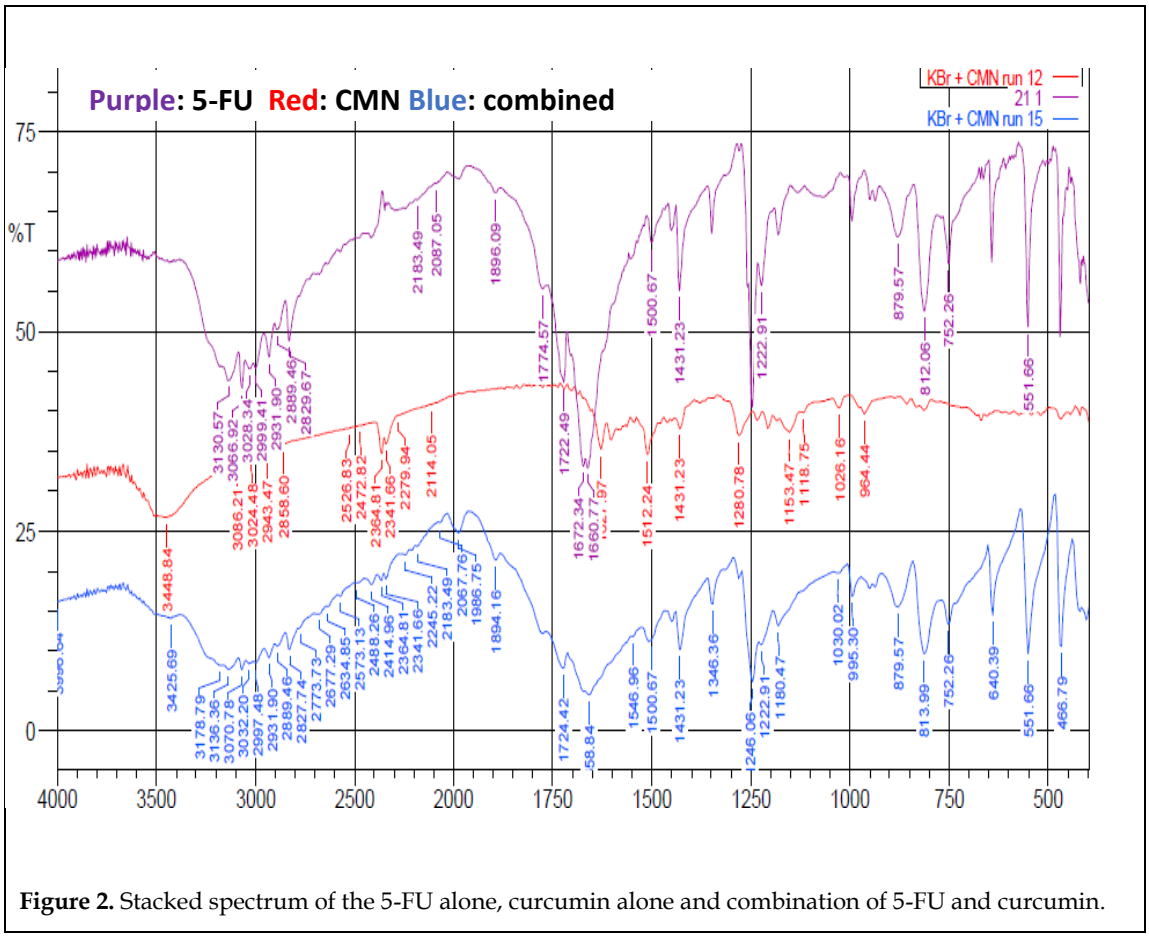


Figure 2. Stacked spectrum of the 5-FU alone, curcumin alone and combination of 5-FU and curcumin.

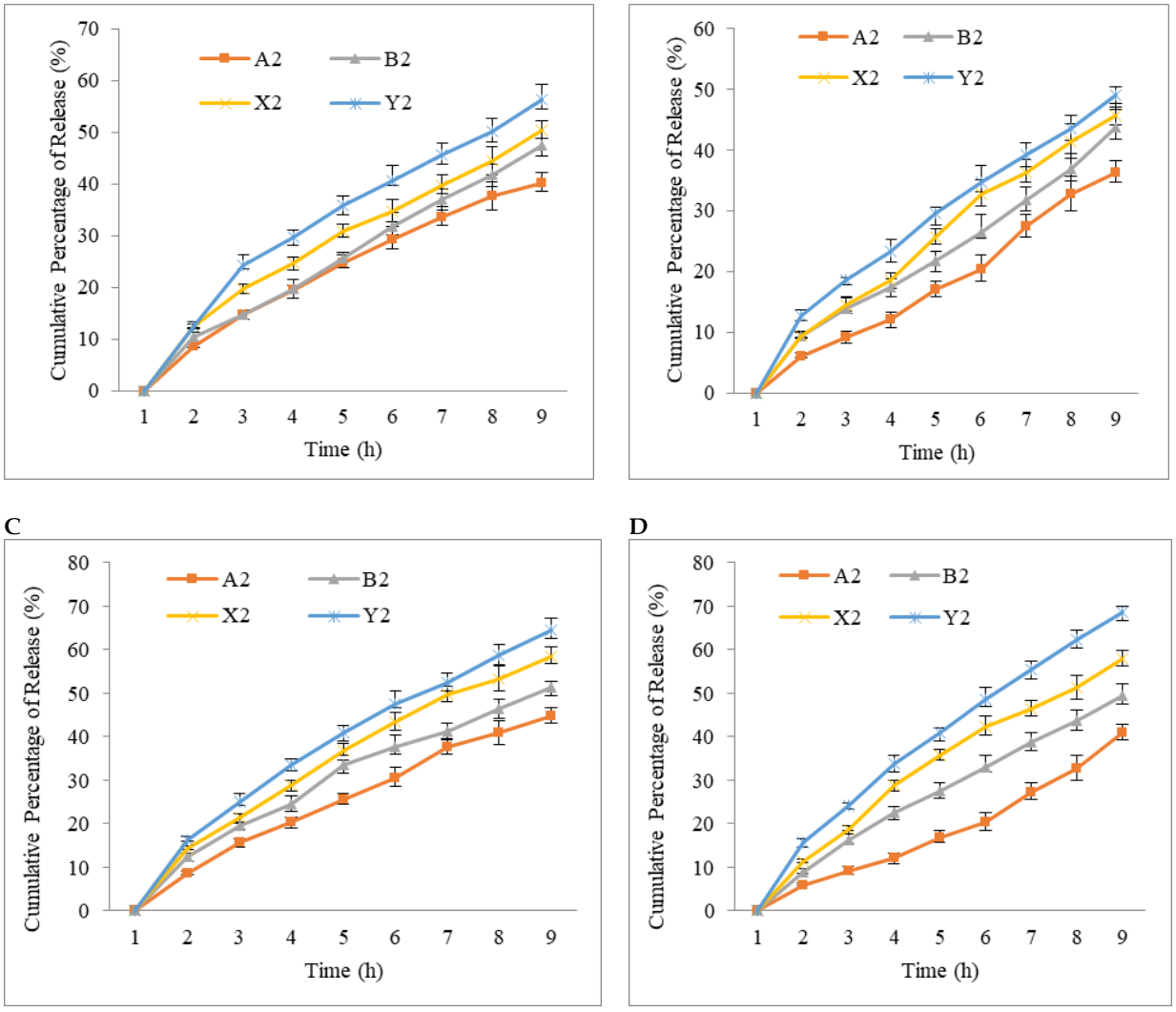
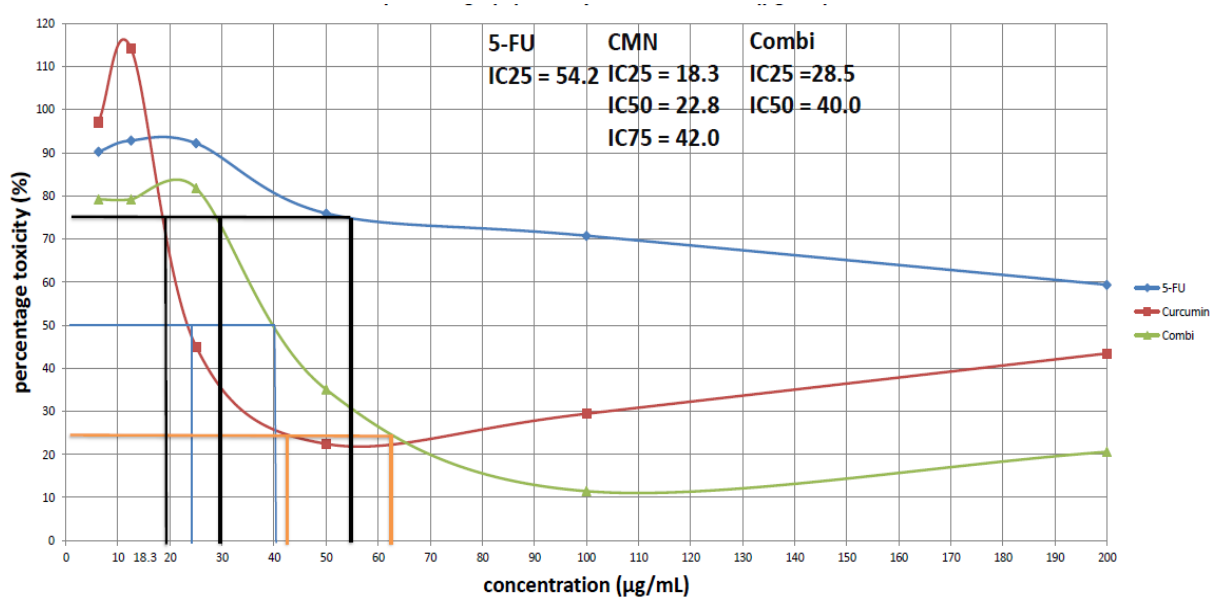


Figure 3. (A) Dissolution profile of 5-FU with PBS; **(B)** dissolution profile of 5-FU VSF; **(C)** dissolution profile curcumin with PBS; **(D)** dissolution profile curcumin with VSF.

Particle size

The vesicle size distributions were homogeneous and were within the acceptable limits for Tween 80 based formulations. The zeta potential of the polysorbate LC formulations with higher negative values incline to resist vesicular aggregation consequently does not produce particle-particle aggregation (Scholes et al., 1999; Zhang et al., 2010). This phenomenon may be due to the low molecular weight of Tween 80 leading to low zeta potential values. This is evidenced by the lesser particle size of Tween 80 than higher ratio formulations (Rajabalaya et al., 2016). The particle size is influenced by the synthetic drug, 5-FU, When FU ratio increases the particle also increases, which may be due to the lipophilic nature of the drug. The size of the vesicle and its distribution in the formulations are of paramount importance for the combination drugs which determines the drug delivery profile of the formulation (Plessis et al., 1994).



Combination Index (CI) at specific Effective Dose (ED)

Combination	ED_{50}	ED_{75}	ED_{90}	ED_{95}
5-FU and Curcumin	0.64804*	0.72285*	0.81759**	0.88973*

Figure 4. Graph of % toxicity *vs.* concentration (µg/mL).

The results are presented as mean ± SEM. *p<0.05, **p<0.01, ***p<0.001 indicate significant differences with respect to base valor. 5-FU: 5-fluoracyl; CMN: curcumin; Combi: Combination of 5-FU + CMN.

Environmental Scanning Electron Microscopy (eSEM)

eSEM evidently demonstrated that the lower concentrations of Tween 80 containing LC formulations formed very small spherical shape with compared to higher ratios of components of 5-FU and curcumin. In the lower concentration of Tween 80-based formulations, the vesicles have lesser spherical shape compared to higher concentrations formulations. The repulsion between the surfactant head groups lead to higher curvature in the micelles, forming spherical vesicles (Bengt et al., 2014). The higher value of zeta potential observed in the polysorbate based gels indicating the better dispersion of LC vesicles in the formulations when diluted with the distilled water (Shustova et al., 2011). It was observed that gels with lower concentration of drug produced spherical micellar structures. Moreover, micelles with higher concentration of the drug content showed black dotted on the surface that indicates the distribution of hydrophobic drug, either 5-FU or curcumin solubilized in hydrophobic chain molecules. Thus, this postulates that higher amount of drug is present in the micelles.

In eSEM, the prepared sample is placed directly on the sample holder, where the sample is evaporated resulting in the aggregation of the sample thus making the size bigger. While in zetasizer, the sample is diluted, thus leading to smaller particle size. Although the particles form micelles, it is not confirmed whether the micelles are normal or reversed phase micelles. Thus, a more powerful microscope such as cryo-Transmission Electron Microscopy (cryo-TEM) need to be use in order to observe the micelles at higher magnification to differentiate the micelle type.

Fourier transforms infrared spectroscopy (FTIR)

Based on the stacked spectrum, all bands from 5-FU alone and curcumin alone were present in the spectrum of the combination of the drugs (Fig. 2). This indicates that there was no chemical interaction between the chemicals when mixed together. The individual components curcumin, 5- FU and Tween 80 combination mixtures demonstrated compatibility of the surfactants with the drug in the formulation as the spectra was without significant peak shifts. This indicates that no chemical interaction between the drug and the surfactant. There were few characteristic peaks of the drug, which were overlapping in the region as that of the surfactants possibly due to the encapsulation of the drug between the layers.

Dissolution

The PBS with pH of 7.0 – 7.4 represents the vaginal pH during menopause, and vaginal simulated fluid (VSF) with pH of 3.5 – 4.9 represents vaginal pH during adult life. Dissolution study was carried out in both of these simulated fluids to have wide range of applications for the liquid crystal gel formulation.

LC formulations showed that they were a good delivery vehicle throughout this research. They were suitable to be used as topical drug delivery system since they have mucoadhesive properties, which increase the absorption of drugs when applied topically and were able to incorporate both 5-FU and curcumin. The consistent drug release demonstrates the stability of both the drugs used. Furthermore, in the dissolution study, the drug release increases with time indicating sustained drug release profile and good percentage of drug dissolution (Ahn et al., 2010). It is also seen in both buffer systems, namely PBS and VSF, that the delivery of the drug is increasing and did not reach plateau, which indicates that there is drug still entrapped in the liquid crystal system. This signifies that this LC gel system has a sustained release profile.

The dissolution behaviors show an almost similar pattern with other drug and surfactants ratio; such as when the drug and surfactant concentration increase the percentages of dissolution also increases. It may be due to higher surfactant influencing drug dissolution, which may be due to improved physicochemical properties of drug like solubility, liquid crystalline forms, particle size, and diffusivity of the compound (Jamzad and Fassihi, 2006). Dissolution study using VSF with the same drug release pattern exhibits compatibility of the LC system in both pH environments. The higher curcumin percentages of dissolution compared to 5-FU, may be due to two-fold factors; firstly, the ability of the surfactant to solubilize the curcumin efficiently in the dissolution medium and secondly, the optimal particle size enhancing dissolution in both the pH mediums. This shows that the formulation is suitable to be used in both post-menopause patients and adult patients (Lara et al., 2005).

Drug efficacy study

The drug efficacy study indicates that 5-FU when given alone requires a higher concentration to produce its effects. Based on Chou (2006), the combination index (CI) value was 0.64804 and the CI value were in the range of 0.3 – 0.7 and showed synergism between 5-FU and curcumin. Another study showed that the CI value for both ED_{75} and ED_{90} were in the range of 0.7 – 0.85, which indicate moderate synergism while CI value for ED_{95} showed slight synergism between the two drugs (Liu et al., 2017). Another study by Du et al. (2006) only focused on the synergism of 5-FU and curcumin.

The current drug efficacy study shows synergism at ED_{50}, for the combination of 5-FU and curcumin. The combination of the drugs is safe to be applied and with further study, the dose of 5-FU may be reduced while increasing the dose of curcumin. With this, the therapeutic effect may be maintained, while the side effects of 5-FU may be reduced (Seong-Ho A, 2010).

CONCLUSIONS

The present study demonstrates that the combination of 5-FU and curcumin in liquid crystal system is stable with appropriate pH that is suita-

ble for both adult female and post-menopause female. It also has suitable physicochemical properties that fit the criteria to be used topically in the vagina. The sustained drug release profile in the dissolution study shows that the LC system releases the required amount of drugs for a very long time. The combination of 5-FU and curcumin is suitable to be used on cervical cancer HeLa cell line.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Contribution	David SR	Refai SA	Yain KR	Mai C-W	Das SK	Rajabalaya R
Concepts or ideas	x			x		x
Design				x		x
Definition of intellectual content	x					x
Literature search		x	x			
Experimental studies	x	x	x	x	x	x
Data acquisition	x	x		x	x	x
Data analysis	x		x	x		x
Statistical analysis			x	x	x	
Manuscript preparation	x	x				x
Manuscript editing	x					
Manuscript review	x	x	x	x	x	x

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