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An effective method for the development of the liposomal nanoformulations of docetaxel

Angham M. Abed
University of Babylon, College of Medicine, Department of Pharmacology / Iraq
Email: anghammohsen89@gmail.com

Majed K. Abbas
University of Babylon, College of Medicine, Department of Pharmacology / Iraq

Amjed M. Oda
University of Babylon / College of Basic Education, Department of chemistry, Iraq

***Abstract*---**Docetaxel has been regarded as among the most effective chemotherapeutic medications in recent decades, but its low solubility in water with systemic toxicities have made it difficult to use in clinical trials. Nanotechnology has supplied docetaxel with innovative drug delivery technologies in recent decades, allowing it to increase their water solubility and lessen adverse effects. Liposome was successfully used for encapsulation the Docetaxel in variant conditions and optimized to synthesis lecithin liposome and loaded one. The thin film hydration method was demonstrated for nanocapsules. The synthesized Docetaxel-liposome formula was investigated by SEM, TEM and zeta potential used for liposome stability. Encapsulation and loading efficiency was determined and releasing kinetics was studied at pH= 5.5 and 7. The kinetics models were studied included: zero-order, first-order order, Higuchi, and Korsmeyer Peppas model. The docetaxel-liposome was examined for its activity to kill prostate cancer in the cell line method and compared to free docetaxel (pure sample) and pharmaceutical formula. Our formula was very effective against prostate cancer and statistically significance.

***Keywords*---**prostate cancer, nanotechnology, drug delivery system, liposome, docetaxel.

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***Corresponding author**: Abed, A. M.; Email: anghammohsen89@gmail.com*
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Introduction

Nanomaterials for cancer treatment are already a reality, bringing plenty of the new tools and possibility , ranging from the earlier diagnosis and better imaging to more effective, efficient, and targeted anticancer medicines [1]. Cancer is a worldwide health issue that is regarded as one of the important causes of morbidity and death [1]. Tumor cells are unique in that they are heterogeneous, versatile, and capable of adapting to anti-cancer settings [2]. In recent decades, several ways for increasing therapeutic influence and minimizing tumor recurrence have been investigated, including medication delivery utilizing nanomaterials, which is one of the most promising methods for cancer healing. Liposomal, inorganic, polymer, dendrimer, and micelle nanoparticles have been designed to increase drug load, extend half-life, control drug release, reduce adverse effects, and lower costs [4,[3]. Liposomes, which are made up of phospholipids, have been researched for decades to see if they may be used in therapeutic formulations [4] Liposomes have significant benefits and prospective capabilities as compared to other nanocarriers in terms of delivering a variety of generally ineffective pharmaceuticals by modifying their physicochemical properties and biodistribution, as well as minimizing drug toxicity [7-10]. A Docetaxel (Doc) component stimulates cell growth by activating a supported square at the metaphase-anaphase boundary during cell division, altering the microtubular arrangement essential for mitotic cell division [5]. Doc also prevents microtubule depolymerization back to tubulin, resulting in cell dissatisfaction [6]. The high measurement boundary can be lifted if the drugs are intended to be more site-specific and focused on rather than the current regular intravenous (IV) distribution. For example, focused nanohybrids based on titanate nanotubes coupled with Doc proved to have superior cytotoxicity and were less harmful than free docetaxel against human LNCaP prostate cancer cells [7].

Experimental section

Materials

The Chemical used in our study include

The chemicals that used in experimental part are: chloroform (THOMAS BAKER, India), Cholesterol (Dyc, Korea), Docetaxel vial (Sanofi Aventis ,France), Lecithin (Sigma Aldrich, USA), Methanol(THOMAS BAKER, India) and Pure Docetaxel Powder (Sigma Aldrich, USA).

Instrumentation

The Instrument used in our study include:

Rotary evaporator (Heidolph, Germany), Lyophilizer(Lte scientific ltd, great Britain), Oven (Memmert ,Germany),Probe sonicator (Qsonica U.S.A), Incubator (Memmert ,Germany) and Uv-spectrophotometer (Karl kolb, Germany).

Procedure

Preparation of docetaxel loaded liposomes

The method used to prepare liposome was the thin film hydration method, we prepare the stock solution (10 mg/ml lecithin,10mg/ml cholesterol), then we take (1.1 molar ratio) from them and mix briefly, to speed up the evaporation process we used rotary evaporator for 20 mint, after completion of the evaporation process, cover the hole of the flask by a piece of parafilm to prevent dust entering and make a small hole in parafilm by using needle, this flask stay overnight in a desiccator to eliminate any remaining organic solvent.

Hydration of the Thin Film and drug encapsulation

To the dry lipid film, 2 mL of phosphate buffer saline was added. The dried thin film was hydrated in the aqueous solution containing the drug [8], Because docetaxel is a poorly water-soluble drug, it is firstly dissolved with alcohol[9], Vortex the solution three times for 10 seconds to suspend the lipid materials in the solution. To help the lipids suspend in the solution ,sonicate them for 15 seconds in a water bath sonicator. then allow the suspension to stand at 4 °C overnight to efficient hydrate all the lipid materials. After the preparation of liposomes which contain mostly MLVs, to decrease the particle size ,we sonicated the liposome . then it extruded by using polycarbonate membrane (size 100 nm).The sample will have a semi-translucent appearance., then transfer this sample from the syringe to a storage vial. Store the liposome sample at 4 degrees Celsius[10].

Results and Discussion

A-Characterizations of Nanoparticles

The liposomal docetaxel has been dispersed in distilled water and sonicated at 40% AMP for 2 minutes then used for characterization.

Scanning electron microscopy (SEM)

Scanning electron microscopy (SEM) is a widely used technique for imaging the microstructure and morphology of materials [11] . The liposomal nanoparticles showed spherical morphology and the size of Liposomal docetaxel approximately 20 nm in range (fig 1). The liposomes all possessed a fine spherical form with a largely monodispersed size distribution, which can be seen in SEM pictures, confirming the size distribution measurement tests. (Fig. 1 &Fig. 2) .

Liposomes in our study were 28 nm in size, while liposomal docetaxel was 20 nm. Several earlier investigations using liposomes of various mean sizes suggest that vesicles in the 20–200 nm range are more successful at extravasating and accumulating inside tumor tissue and inflammatory areas. As a result, the Docetaxel-loaded nanoparticles generated in this study were in the size range that favors therapeutic effects for tumor treatment. [9].

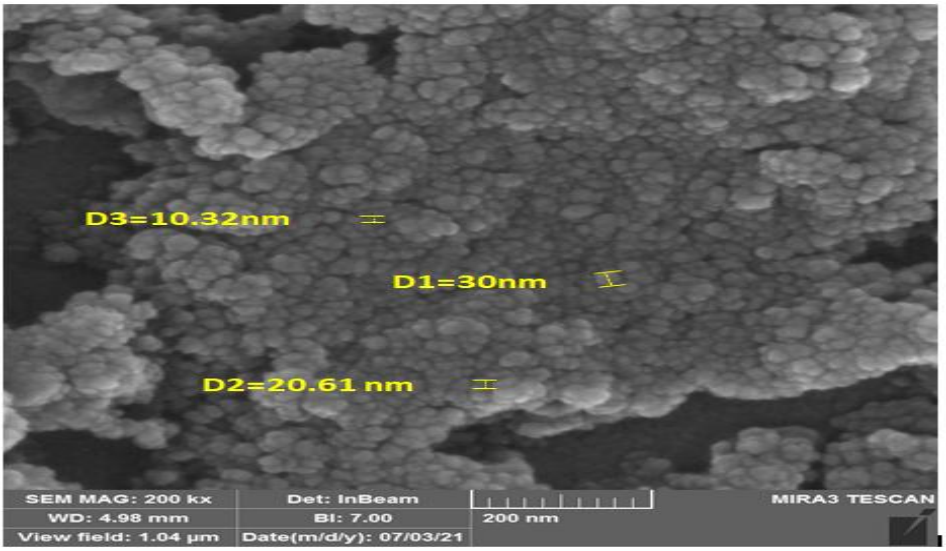

Figure 1:SEM of Liposomal Docetaxel

Transmission Electron Microscopy (TEM)

Liposomal docetaxel's surface morphology was shown to be spherical in shape with a reasonable sized distribution (Fig. 2). TEM revealed that the drug-loaded liposome's surface shape was spherical. The particle size can also be validated using a TEM picture, which revealed that Liposomal docetaxel has a particle size of 14 nm (Fig. 3).

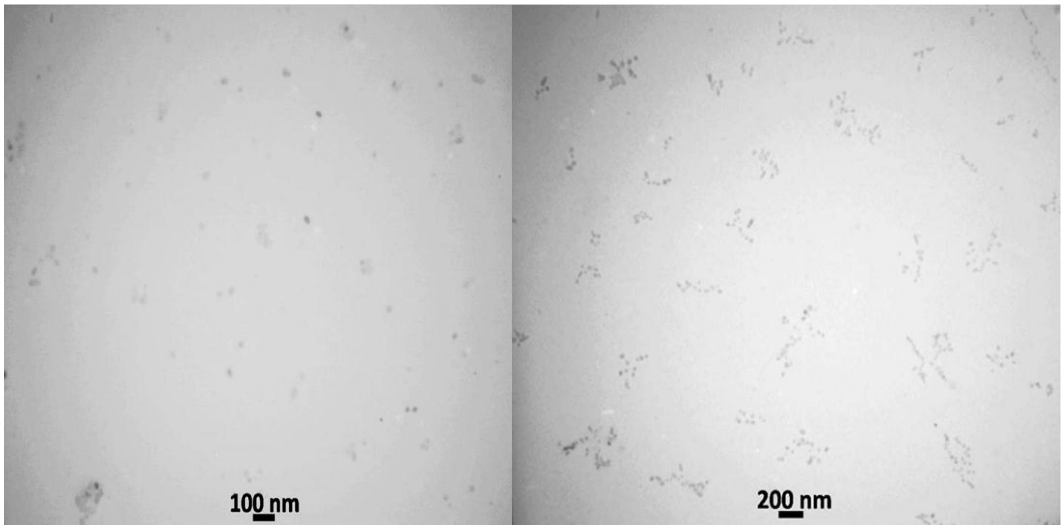

Figure 2: TEM of L.Doc at 200nm,100nm

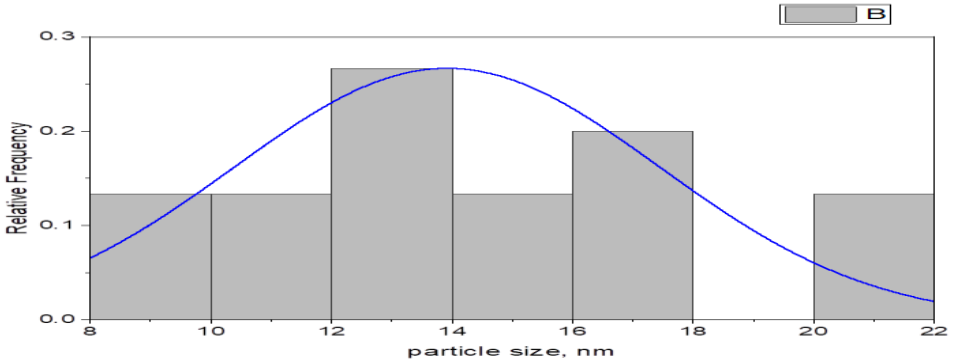

Figure 3: Size distribution of liposomal docetaxel

Zeta Potential

The Zeta potential of the liposome and liposomal docetaxel was -28.3 mV shown in fig.4. The zeta potential determination of nanocrystals is an important characterisation approach for estimating the surface charge that may be used to better understand the physical stability of nanosuspensions [12] . ZP values are typically in the range of +100 to -100 MVA [13]. Due to electrostatic repulsion of individual particles, nanocrystals with a large positive or negative zeta potential imply good physical stability of nanosuspensions. The value of ZP in our approach is -28.3 mV (Fig 4), which is regarded optimal for effective nanodispersion stabilization [14].

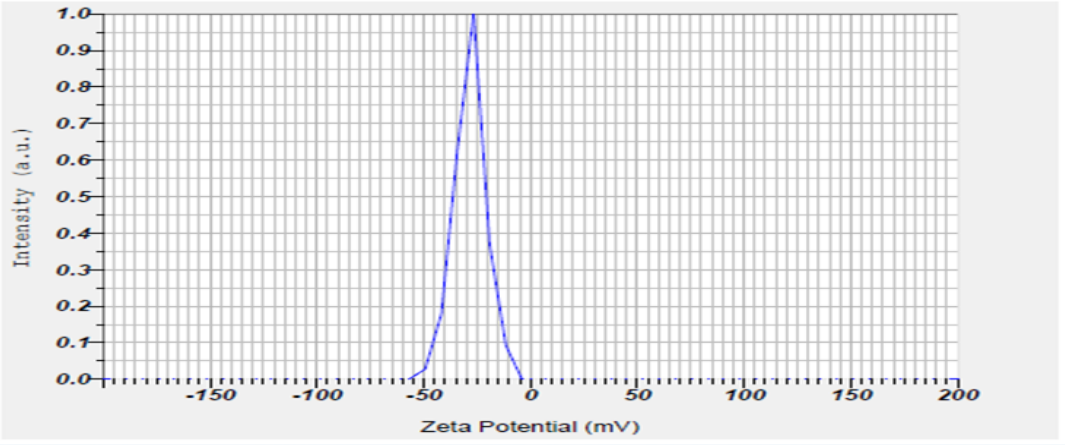

Figure 4:Zeta potential of liposomal docetaxel

Stability

The preparations were stored at refrigerating (4 °C) for 3 months and the zeta potential of the formulations was measured(-36.5 mv) which is shown in (figure5) and compared with those at the time of preparation, this value means the nanoformulation is still physical stable according to [15], This could be due to the presence of cholesterol, which has a stabilizing influence against liposome aggregation and fusion [16].

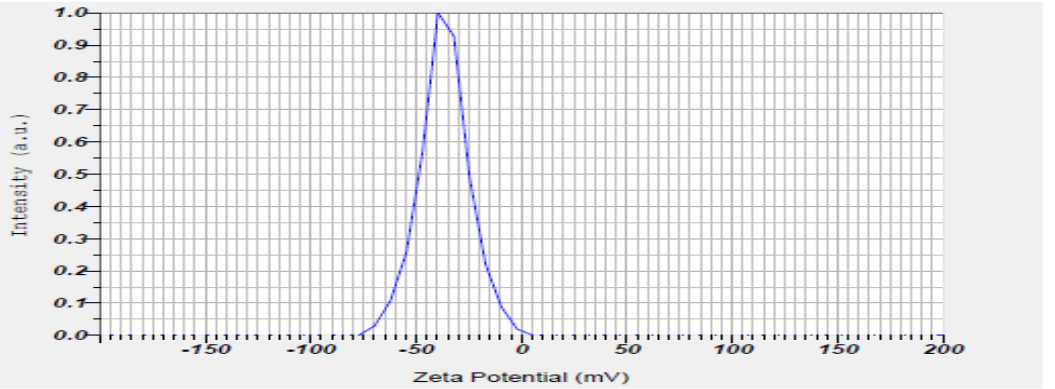

Figure 5:Zeta potential of liposomal docetaxel after three months.

Drug Loading (DL)

The following equations were used for calculation of drug loading percent:
% Drug loading (% DL) = (amount of encapsulated drug / amount of lipids) × 100
The amount of encapsulated drug was calculated by measuring the amount of free drug in the spectrophotometer, then comparing this absorbance value with the concentration in calibration curve, the value of %DL was equal to 79.54%.
DL% depends on the composition of the lipid matrix and its crystalline state[17].
The amount of DL% was equal to 79.54% which consider a high amount in drug loading and a sign for successful remote loading and good encapsulation of Doc into the liposomes [8].

The Encapsulation Efficiency (EE)%

The **Encapsulation** efficiency (EE) was calculated according to the following equation:

$$\text{EE}(\%) = \frac{\text{amount of Doc entrapped in liposomes}}{\text{amount of total Doc applied in the preparation}} \times 100$$

We prepare liposome with different amount cholesterol and phospholipids (Table 1) to measure encapsulation efficiency and chose the better ratio to make further investigation.in our experiment we take 1000µg of docetaxel and the amount of docetaxel entrapped in liposome was obtain by spectrophotometer to measure its absorbance ,then calculated its concentration according to calibration curve which equal to 23.49µg which mean the amount of Doc free in solution equal to (1000-23.49 =187.92) and the amount of encapsulation was calculated according to above equation .the amount of Doc entrapped in liposome equal to (total amount of feeding drug minus concentration of free docetaxel in solution.

Table 1:calculation of EE% for different amounts of cholesterol and phospholipid

Molar ratio	Cholesterol amount(Ml)	Phospholipid amount (Ml)	Con.of free docetaxel (mg)	EE%
1:1	1330	670	23.49	81.2
0.25:1	670	1330	23.97	80.8
0.5:1	1000	1000	30	76
1:14	250	1750	23.97	80.8
0:1	0	2000	30.1	75.9
2:1	1600	400	28.96	76.8

EE(%)=81.82% (molar ratio 1:1 provided optimal encapsulation efficacy)[10].
The most important outcome was the results of measurement of liposomal encapsulation efficiency EE(%)=81.82% for docetaxel nanoliposomes, which consider the higher percent of encapsulation according to [18], who ranging the high efficiency of docetaxel encapsulation, ranging from 68.53 percent to 99.45 percent, with the lipid to drug ratio accounting for the majority of this percent .

In vitro drug release

In vitro drug release profile using dialysis, the bag was measured by the spectrophotometer at 227 of encapsulated docetaxel at pH 5.5 and 7 has been observed after 24, 48, 72, and 96 hours illustrated in Fig.6.

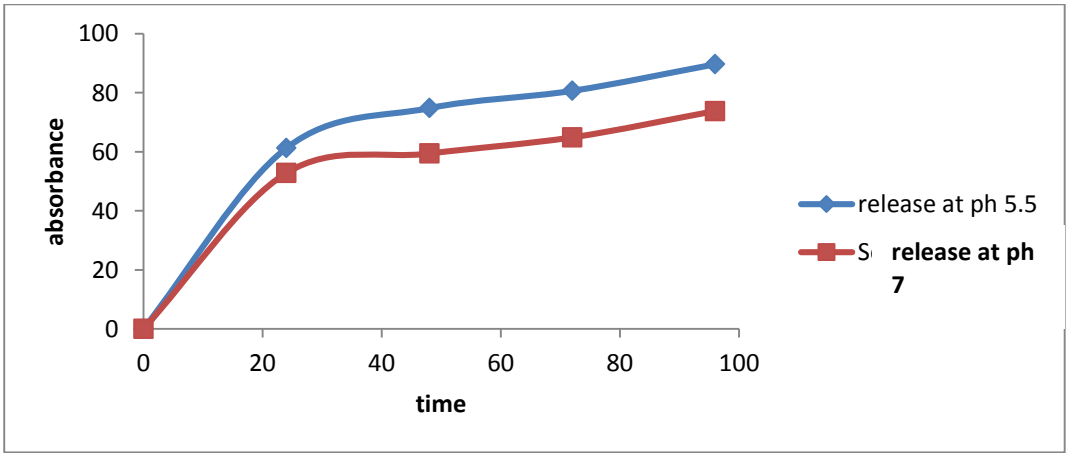

Figure 6:Liposomal Docetaxel release profile at ph 7 and 5.5 after 24, 48, 72, and 96 hours

In our study, We chose the PH of prostate 5.5 to analyze the release pattern because the drug release was higher at pH 5.5,(Fig .6). So because the acidic extracellular and intracellular microenvironment of tumors would be predicted to increase drug release, greater drug release at acidic pH is favorable for drug delivery [19].

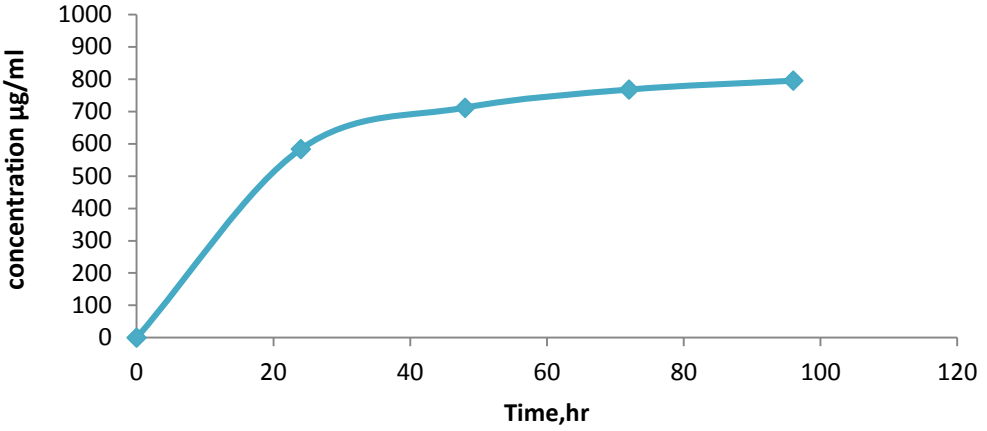

Figure 7: concentration of drug released during 24,48,72,96 hr

Four drug release kinetic models, including the zero-order kinetic model, first-order kinetic order, Higuchi model , and Korsmeyer Peppas model, were utilized to assess the in vitro release patterns of Doc-Lips. The zero-order kinetic model (Fig. 8 A) was a relationship between time and cumulative percent drug release that could be used to define the process of releasing a constant drug from a drug delivery system. Meanwhile, the first-order kinetic model (Fig. 8 B) represented the link between time and the log cumulative percent of drug left. The concentration-dependent mechanism of drug release was evaluated using this model The Higuchi model (fig. 8 C) was a relationship between the square root of time and cumulative percent drug release that was used to determine whether the primary drug release mechanism was diffusion-controlled or not. Finally, the Korsmeyer-Peppas model(Fig. 8 D) was a relationship between time and log cumulative percent drug release that aided in the understanding of drug dissolution mechanisms from the matrix. Using Microsoft Excel and equations (1), (2), (3), and (4), graphs of the zero-order, first-order, Higuchi, and Korsmeyer-Peppas models were created, and the rate constant and correlation values were calculated using a linear regression fit [20].

$$C = k^{\circ}t \quad (1)$$

$$\log(100 - C) = -\frac{k_f t}{2.303} \quad (2)$$

$$C = k_H \sqrt{t} \quad (3)$$

$$C = k_K t^n \quad (4)$$

where is C the cumulative % drug released at the time, K0 is the zero-order rate constant, Kf is the first-order rate constant, KH is the Higuchi dissolution constant, Kk is the Korsmeyer-Peppas constant, and n is the exponent that refer to a particular diffusion mechanism.

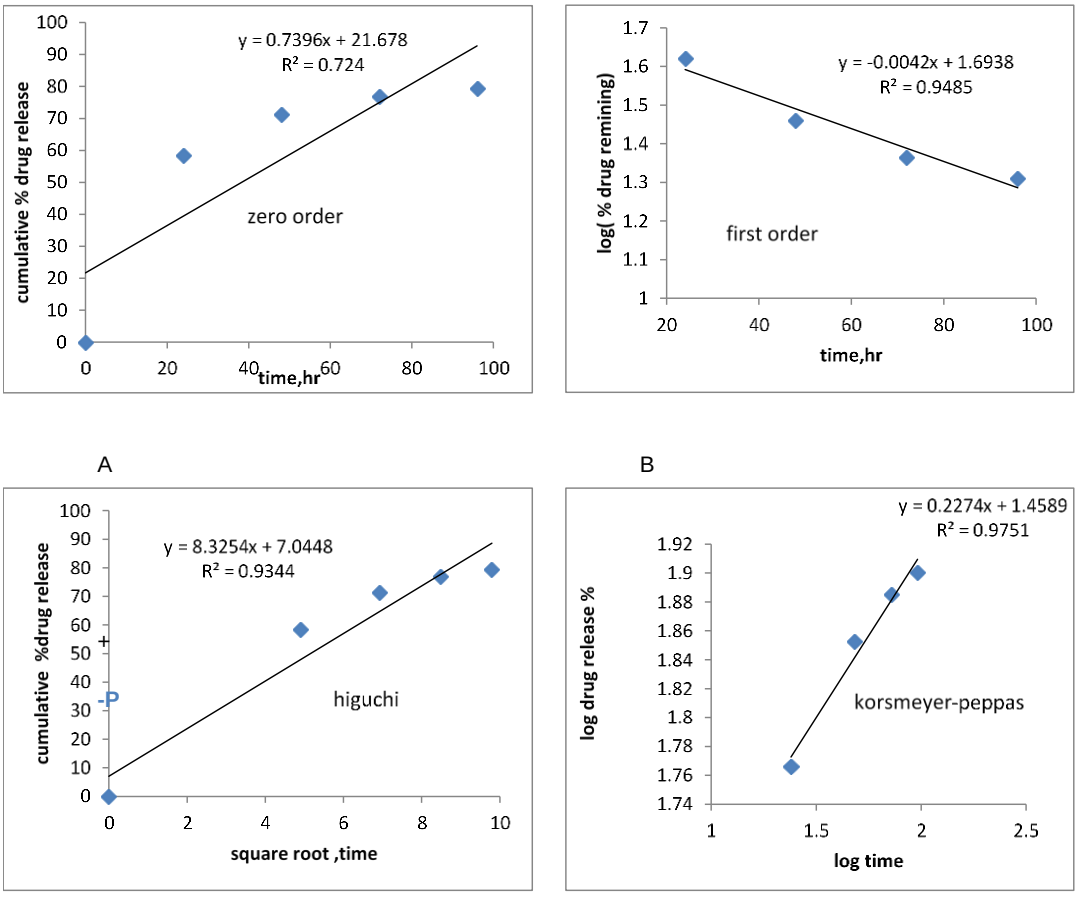

Figure 8:invitro release kinetic models

As shown in figure 7, At ph 5.5 the *in vitro* drug release profile of L.Doc has been observed after 24, 48, 72, and 96 hours, The Ph 5.5 represents the favorable ph of prostate cancer (Ceylan, *et al.,* 2020). sustainable slow-release has been obtained where after 24 hours the absorbance was 61.25, after 48 hours the absorbance was 74.81, after 72 hours the absorbance was 80.68 and after 96 hours the absorbance was 89.67. The well-encapsulating core of liposomes may be responsible for the sustained and delayed release of docetaxel. This allows for continual combat against cancer cells, resulting in a reduction in cancer cell viability (Cheng, K *et al.,* 2018). generally, the utilization of liposomes for the administration of anticancer medicines for docetaxel encapsulation can prolong and regulate release profile (Gan *et al.,* 2010).

The pattern of drug release from the formulation was best fitted with the Korsmeyer–Peppas kinetics(R^2 = 0.9741) (Fig.8 D) which exhibits the involvement of strange diffusion which is controlled by more than one parameter, the resulting agreement with[21]study. Because lipid bilayers are maintained by cholesterol, the data shows that docetaxel takes longer to be released when encapsulated in liposomes. Liposomes could thus be used to create a depot effect. The above

findings suggest that our liposomal formulations satisfies the criteria for a successful drug delivery system [11]. Our liposomal docetaxel release profile shows the typical sustained and prolonged drug release (as seen in Fig.7).

In vitro cytotoxicity

We analyzed the result of LNCaP prostate cancer cells by using the different formulations of docetaxel which include liposomal docetaxel(L.Doc), pure docetaxel, and formulated docetaxel (TAXOTERE), to compare their activity of them at different periods of incubation for 24,48 and 72hr with different concentrations (500,250,125,62.5,31.25 and 15.62)µg/ml.Fig 9,10 and 11 .

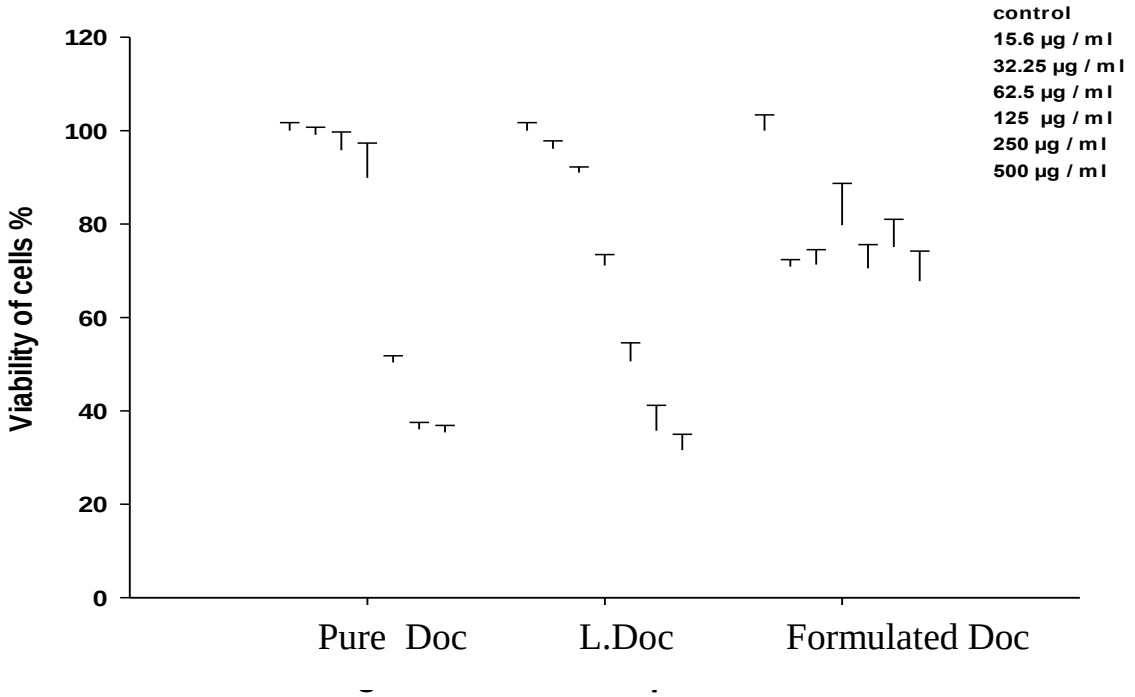

Figure 9 : drugs (Pure docetaxel, liposomal docetaxel and formulated docetaxel) After incubation periods 24hr

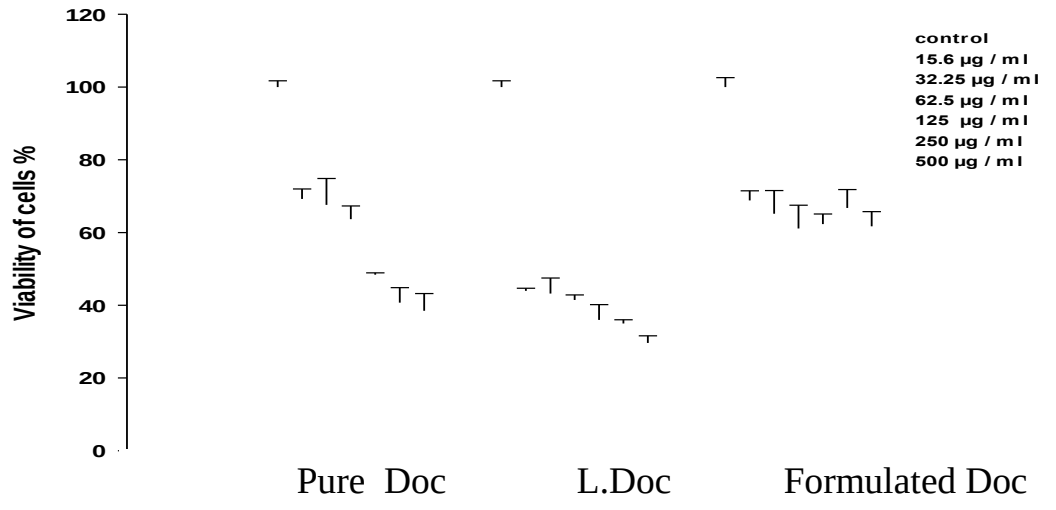

Figure 10: drugs(pure docetaxel, liposomal docetaxel and formulated docetaxel) After incubation periods 48hr

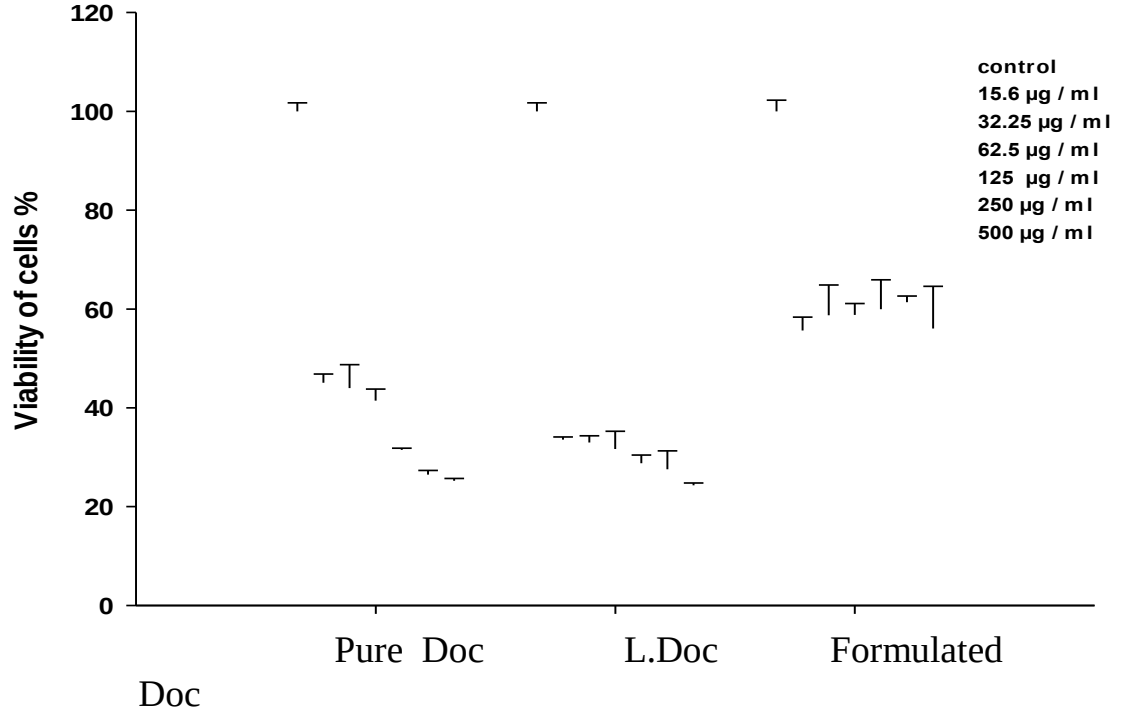

Figure 11: drugs (pure docetaxel, liposomal docetaxel and formulated docetaxel) After incubation periods 72hr

The MTT assay method was used to assess the viability or anti-proliferative effect of free Doc, L-Doc, and Taxotere on prostate cancer cells in vitro. The figure of percent cell viability vs. concentration reveals that the death rate of cells increased as the concentration of all forms of Doc increased. As illustrated in Figs. 9, 10 and 11, L-Doc mediated cellular death was found to be greater than that of cells treated with a pure drug solution and formulated docetaxel, respectively. this result agreement with [21] study.

Conclusion

In conclusion, Doc-loaded liposomes were synthesized and their physicochemical properties studied. L. Doc exhibited a pH-dependent release pattern, which would be advantageous for tumor cell selectivity. Furthermore, increased drug release in acidic environments may inhibit P-GP activity and lessen P-GP–mediated drug resistance. Overall, Liposomal nanoparticles have been shown to give a new platform for cancer treatment.

The cell viability of liposomal nanoformulations treated against LNCaP cells decreases over time, demonstrating that the nanoformulations slow down LNCaP cell proliferation. As a result, the produced liposomal Doc nanoformulations might be used as a viable and alternative drug delivery mechanism for prostate cancer treatment, resulting in better therapeutic efficacy while using less drug.

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