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Design and Evaluation of Dry Powder Inhalation Formulation for Pulmonary Delivery of Docetaxel: A Novel Approach in Non-Small Cell Lung Cancer

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Objective: Cancer remains a significant global health challenge, with escalating incidence rates projected for the near future. Docetaxel, a potent anticancer agent, is difficult to deliver owing to its poor oral bioavailability and the adverse effects associated with current formulations. This study aimed to develop a novel dry powder inhalation formulation of docetaxel for pulmonary delivery utilizing cholesterol and Vitamin TPGS as excipients. Methods: The formulation process involved solvent hydration and lyophilization to produce polymeric microparticles. Compatibility studies using Fourier-transform infrared spectroscopy demonstrated no interaction between Docetaxel and excipients. Results: Evaluation of the developed formulations revealed that the moisture content was within 2.0%, angle of repose indicated good flow properties, and high drug content was between 95.00% and 105.00% with encapsulation efficiency from 75.91% to 89.18%. Scanning electron microscopy confirmed a spherical morphology with a particle size of less than 6 µm. The in vitro release studies showed sustained release profiles, with formulation F12 exhibiting the highest release (86.18%) at 8 h. Further, the cytotoxicity and cell cycle studies in A549 cells were evaluated, and the IC50 required for F12 was 47.54 and 54.69% of cells were arrested in 24 h, respectively. Conclusion: An Optimized method was developed using tocopherol and cholesterol, which is scalable to large production, and all the results fall within the range of pulmonary Drug Therapy.

Keywords: Docetaxel, Dry Powder Inhaler, Solvent Evaporation Method, Pulmonary route, cholesterol.

1. Introduction

Cancer is not a new disease, and it has been known for centuries. The International Agency for Research on Cancer said, there will be 12.4 million additional cancers, 7.6 million die from the disease, and 1.2 billion individuals living with cancer five years following their original diagnosis. Males are more commonly affected by non-small cell lung cancer in terms of both illness incidence and death [1,2]. The world's population is expected to grow and age over the next two decades. As a result, the incidence of Non-Small Cell Lung Cancer in low- and middle-income countries will continue to increase worldwide. By 2030, it is conceivable that 26.4 million new cancer diagnoses and 17.0 million cancer deaths will occur annually. There is solid evidence that cancer will continue to be a severe public health issue in the near future. Identifying treatments for many types of cancer is the most obvious method to prevent needless deaths [3].

Docetaxel, discovered in France in 1980, belongs to the toxoid class of cytotoxic agents. A novel semi-synthetic anticancer drug, docetaxel, was developed from Bacatin III, the needles of the European yew taxus baccata[4]. Docetaxel interacts with tubulin, which triggers its polymerization and promotes the formation of sturdy microtubules, resulting in a unique mechanism of action. Conversely, drugs lead to the depolymerization of microtubules in vivo, thereby hindering their function by producing the opposite effect; they excessively stabilize the structure of microtubules. This restricts the ability of cells to use their cytoskeleton adaptably. Furthermore, docetaxel selectively binds to tubulin subunits. Tubulin is a fundamental component of microtubules, and docetaxel binding reinforces these building blocks. The resulting microtubule/DTX complexes were stationary. This has detrimental consequences for cell function because continuous modification of microtubule length (dynamic instability) is necessary for the cell to serve as a transportation route[5,6,7,8].

The US FDA approved DCX for breast cancer treatment in 1996. In 1999, docetaxel was approved by the Food and Drug Administration (FDA) as the first drug for second-line non-small cell lung cancer (NSCLC) therapy and was subsequently approved for first-line use[9,10,11].

The most common method for delivering DCX is the intravenous (IV) route, where the drug solution is injected directly into the vein and spreads quickly throughout the body via the bloodstream[12,13]. Compared with other delivery routes, such as oral[14], intraperitoneal[15], transdermal, and rectal, parenteral administration of DCX has demonstrated a higher area under the curve (AUC) in the plasma[16,17,18]. However, this method also led to increased accumulation of DCX in the liver and heart after 15 min of administration[19,20].

Delivering DCX orally is a challenge due to its low bioavailability, extensive first-pass metabolism, and P-GP efflux pumps[21,22]. The pulmonary route is a promising method of administration for lung cancer therapy because the lung has a thin absorption membrane (0.1-0.2 µm) with a large surface area for absorption (~100 m2) and a high blood flow (5 L/min)

that quickly distributes drug molecules throughout the entire body. To be effective, DCX must reach its intended target for pulmonary delivery.[23].

According to the anatomical structure of the lungs, the placement of a drug in various areas depends on its particle size. For example, inertial impaction keeps particles larger than 10 µm in the oropharyngeal region and larynx, while particles ranging from 2 to 7 µm are found in the tracheobronchial area. In contrast, small particles (0.5 - 2.0 µm) will be gravitationally deposited in the respiratory zone (including bronchioles, alveolar ducts, and alveolar sacs), and particles smaller than 0.5 µm will be expelled through exhalation.[23,24,25]. There has been heightened interest in the advancement of DCX nanoparticles for intravenous (IV) and pulmonary delivery. Nanoparticle drug delivery presents several potential benefits, including enhanced serum solubility, extended systemic circulation, controlled release, and targeted delivery. However, there are challenges associated with using nanoparticles for pulmonary delivery due to their small size, which may result in exhalation. To address this issue, the formulation can be nebulized into a colloidal suspension, mixed with micro-sized inert carriers, such as carbohydrates, amino acids, or phospholipids, or embedded in microparticles. The choice of aerosol device used to deliver the drug to the lungs also influences its formulation.[26,27].

The commercially used docetaxel formulation is in liquid form with polysorbate 80, which is reconstituted with ethanol and further reconstituted with a normal saline infusion or 5% dextrose infusion. Unfortunately, Peripheral neuropathy and severe hypersensitivity reactions are two adverse effects of Polysorbate 80[28].

Therefore, the present work aims to develop a novel formulation through the pulmonary system using Cholesterol, Vitamin TGPS, and evaluate the developed formulation.

2. MATERIALS AND METHODS:

Materials:

Docetaxel trihydrate samples were obtained from Cipla Ltd., Bengaluru, India. Cholesterol, Vitamin TGPS & Lactose were procured from Thermo Scientific, Bengaluru, India, Antares Health Products Inc., Bengaluru, India & Meggle Group GmbH, Bengaluru, India, respectively. Ethanol was procured from Rankem, Bengaluru, India. A549 (Human alveolar lung adenocarcinoma cell line) was procured from NCCS, Pune, India.

Method of Preparation:

Polymeric microparticles were prepared by the solvent hydration method[29,30]. The formulation was optimized using the JMP 18 DOE[31] software by setting the maximum and minimum concentrations allowed for human consumption of cholesterol and Vitamin TGPS. DTX (20 mg) was weighed and mixed with the required quantities of cholesterol and ethanol in round-bottom flasks. The flask was continuously vortexed until the organic solvent completely evaporated and a thin film was formed. The obtained film was kept under vacuum overnight to remove the organic content of the polymeric microparticles. In another beaker, a lactose solution was prepared by adding 50 mg lactose, and a known amount of Vitamin TPGS was dissolved in a known amount of water until the solution was clear. The lactose solution

was then added to a round-bottom flask to dissolve the film. The contents were transferred to a beaker and stirred at 600 RPM for 15 min to obtain a uniform mixture. The uniform mixture was lyophilized for 36 h to obtain a dried powder to stabilize the product[32]. The lyophilized powder was then passed through sieve # 500 to increase the flow of the powder[33]. The compositions of the formulations are listed in Table 1.

Table 1: Formulation chart of Docetaxel polymeric dry powder inhalation powder

Ingredient (mg)	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
Docetaxel	20	20	20	20	20	20	20	20	20	20	20	20
Cholesterol	1.875	1.875	1.875	3.75	3.75	7.5	7.5	7.5	7.5	15	15	15
Ethanol	0.16 mL	0.16 mL	0.16 mL	0.16 mL	0.16 mL	0.16 mL	0.16 mL	0.16 mL	0.16 mL	0.16 mL	0.16 mL	0.16 mL
Vitamin TGPS	0.438	1.75	3.5	1.75	3.5	0.438	0.875	1.75	3.5	0.438	1.75	3.5
Lactose	50	50	50	50	50	50	50	50	50	50	50	50
Water	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL	1.5 mL

Drug-excipient compatibility studies using FTIR

The compatibility of docetaxel with the polymer was determined using Fourier transform infrared spectroscopy. A physical mixture of the drug and polymer was prepared in a 1:1 ratio. The Bucker Alpha II instrument (Bucker, Germany) was used to conduct the compatibility investigations. The scan range was 400–4000 cm^{-1} [34,35].

Moisture Content:

The moisture content was identified using Sartorius moisture content, which uses infrared heating with a metal tube heater at 65°C [36].

$$\text{Moisture Content Formula} = \frac{\text{Wet Wt. of the sample} - \text{Dry Wt. of the sample}}{\text{Wet Wt. of the sample}} \times 100$$

Angle of Repose:

One component of interparticulate friction, or barrier to particle movement, is the angle of repose. According to the USP, it is the constant, three-dimensional angle that a pile of material that resembles a cone and is made using a variety of procedures is assumed with respect to the horizontal base. The angle of repose can be computed using the formula below: $\Theta = \text{Tan}^{-1}(h/r)$ where h= powder cone height; r = powder radius [37].

Drug content:

An equivalent quantity of docetaxel (20 mg) was added to a 100 mL clean volumetric flask, 5 mL of methanol was added, sonicated for 5 min, kept aside at room temperature, diluted to volume with the above diluent, mixed, and filter through a 0.45 µn nylon syringe filter [38].

Encapsulation Efficiency

The Lyophilized product was dissolved in water, and the obtained solution was passed through a 0.45 µm filter to separate the unincorporated drug. The obtained solution was subjected to analysis of free drug content[39].

$$\text{Per cent Encapsulation Efficiency} = \frac{\text{Total drug added} - \text{Non-entrapped drug}}{} \times 100$$

Total drug added

Determination of the particle size

A Malvern-Master sizer was used to determine the particle size of the docetaxel polymeric dry powder inhalation powder. Using a technique called Dynamic Light Scattering, commonly referred to as PCS - Photon Correlation Spectroscopy, detects Brownian motion and connects it to particle size. This is accomplished using a laser to illuminate the particles and analyze the variations in the intensity of the scattered light. The size of a particle is determined by its speed of travel, which is random in liquid media. With this understanding of the connection between diffusion speed and size, the size can be ascertained[40].

Scanning Electron Microscopy

Scanning electron microscopy photographs were taken for the prepared docetaxel polymeric dry powder inhaler (F9, F10, F11, and F12) using a scanning electron microscope (ZEISS, at IISc, Bangalore) at specified magnification at room temperature. Photographs, such as size and shape, were used for morphological characterization[41].

In vitro Release – Dialysis Sacs Method

For in vitro release testing, approximately 20 mg of the formulation's docetaxel equivalent was suspended in 3.0 ml of release medium (Phosphate Buffer Saline Solution pH 7.4). The mixture was then placed in a dialysis bag containing a cellophane membrane. Attaching the dialysis bag to the USP type II dissolution device paddle allowed it to be suspended in 250 mL of the release medium. The release medium was kept at 37°C±0.5°C and 50 RPM. A 5 ml sample was removed and replaced with an equivalent volume of the dissolving media at various time points. The absorbance at 232 nm was used to calculate the DTX concentration in the samples [42,43].

Cytotoxicity study:

The cells were incubated for 24 hours in 96 well plate with 200µl of seed cell suspension equivalent to 20,000 cells per well. Appropriate concentration i.e. 6.25 to 100µM/ml were taken as test agents, and the same plates were incubated for another 24 h. 0.5 mg/mL MTT reagent to volume the plates and was wrapped in aluminum foil to avoid light exposure. Following a three-hour incubation period, 100µl of DMSO solution was added after the MTT reagent was removed. Absorbance at 570 nm was measured using an ELISA reader[44]. The following formula was used to calculate cell viability:

Per cent cell viability = [Mean abs of treated cells/Mean abs of Untreated cells] x 100

Cell cycle analysis of A549 cells by flow cytometry

The cells were cultured in a 6-well plate at a density of 2×10^5 cells/2 ml and incubated for 24 h at 37°C in a CO_2 incubator. After aspirating the medium and treating the cells with the necessary concentration of experimental chemicals (IC_{50}) and controls, the cells were incubated in 2 mL of the culture medium for an entire day. Furthermore, 12 × 75 mm polystyrene tubes were used to directly harvest the cells. After centrifuging the polystyrene tubes for five minutes at 30,000 RPM and 25°C, the supernatants were carefully decanted. Then, 1 mL of cold 70% ethanol was added to the supernatant that has been extracted, which

was incubated at -20°C for 30 min. To ensure that the DNA was stained, 400µL of propidium iodide/RNase staining buffer was used. The cells were then incubated at room temperature for ten–20 min in the dark. Additional analysis of the samples was performed using flow cytometry in a PI/RNase solution.

3. RESULTS AND DISCUSSION:

Drug-excipient compatibility was studied using FTIR, and the data are tabulated in figure-1 and table-2 where, different functional groups were identified at various absorption ranges. The results also revealed no interaction between docetaxel and cholesterol, between docetaxel and vitamin TGPS, or between docetaxel, cholesterol, and vitamin TGPS.

Table 2 Comparative statement of compatibility of drug and excipients with FTIR Spectra

#	**Absorption (cm-1)**	**Group**	**Docetaxel**	**Cholesterol**	**Vitamin TGPS**	**Docetaxel + Cholesterol**	**Docetaxel + Vitamin TGPS**	**Docetaxel + Cholesterol + Vitamin TGPS**
1	3550-3200	N-H stretching	3371	3408	3490	3382	3373	3374
2	3000-2840	C-H stretching	-	2930	-	3372	-	-
3			-	2865	2884	2936	2884	2885
4	1870-1705	C=O stretching	1737	-	1737	1817	1737	1737
5			1710	1708	-	1707	1711	1711
6	1440-1310	O-H bending	1495	-	1404	1498	1460	1461
7			1348			1359	1343	1344
8	1310-1020	C-O stretching	1267	-	1279	1253	1276	1275
9			1244	-	-	1232	1241	1242
10			1156	-	1144	1164	1143	1143
11			1116	-	1103	1120	1104	1105
Results						**No Interaction Between Docetaxel & Cholesterol**	**No Interaction Between Docetaxel & Vitamin TGPS**	**No Interaction Between Docetaxel, Cholesterol & Vitamin TGPS**

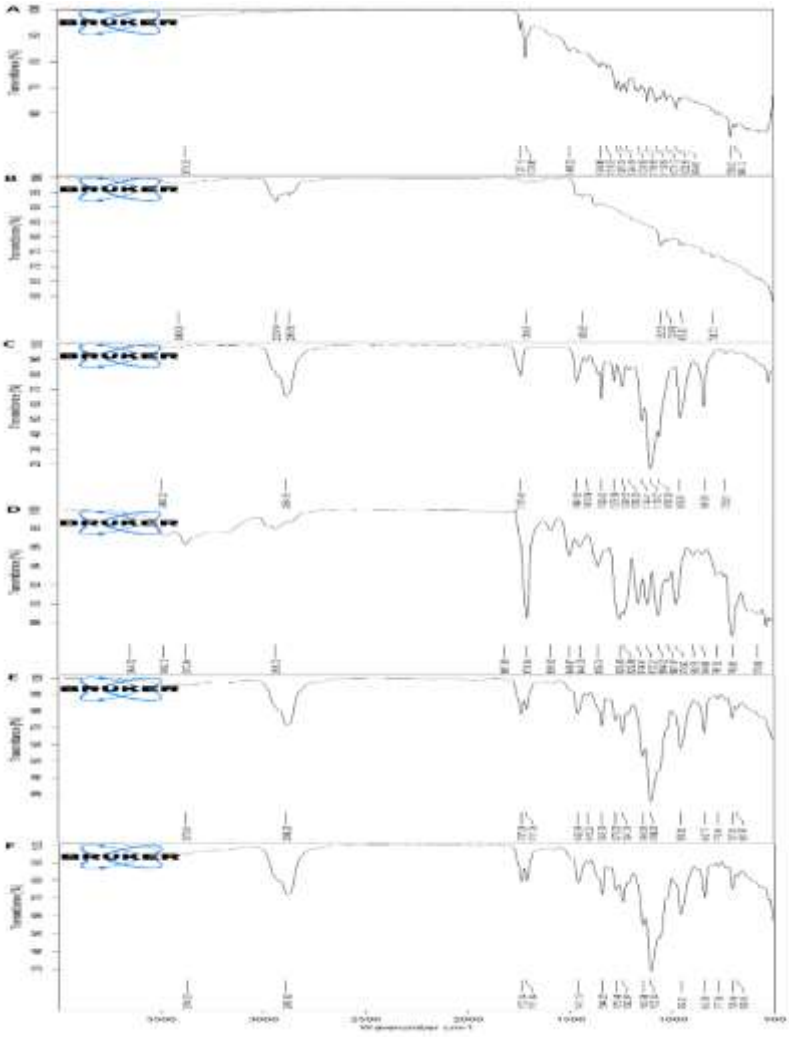

Fig. 1 FTIR Spectra of A Docetaxel, B Cholesterol, C Vitamin E TGPS, D Docetaxel+Cholesterol, E Docetaxel+Vitamin E TGPS and F Docetaxel+Cholesterol+Vitamin E TGPS

The results of the Developed Formulation were evaluated and tabulated in table-3. Drug content was higher with F12 (101.24 %) followed by F1 (100.67 %), whereas percent encapsulation was better with the F12 formulation. SEM analysis revealed the particle size of the formulations, as depicted in figure-2.

Table 3 Evaluation results of Developed Formulation

S/No.	Trail No.	Moisture Content	Angle of Repose(°)	Drug Content (%)	Encapsulation Efficiency (%)	Particle size (D90)
1.	F1	0.58 %	33.70	100.67	25.15	5.182 µm
2.	F2	0.73 %	31.59	99.31	30.25	5.892 µm
3.	F3	1.06 %	30.2	98.63	37.34	4.199 µm
4.	F4	0.90 %	30.2	99.84	58.38	6.041 µm

5.	F5	1.37 %	26.19	97.13	68.68	5.388 µm
6.	F6	1.46 %	29.29	98.28	69.15	5.938 µm
7.	F7	1.26 %	28.97	97.73	70.15	4.059 µm
8.	F8	1.39 %	31.13	99.39	73.15	5.497 µm
9.	F9	1.62 %	25.78	99.94	75.91	5.784 µm
10.	F10	1.41 %	24.65	98.37	77.15	6.099 µm
11.	F11	1.53 %	24.66	99.28	81.25	5.820 µm
12.	F12	1.76 %	23.89	101.24	89.18	5.779 µm

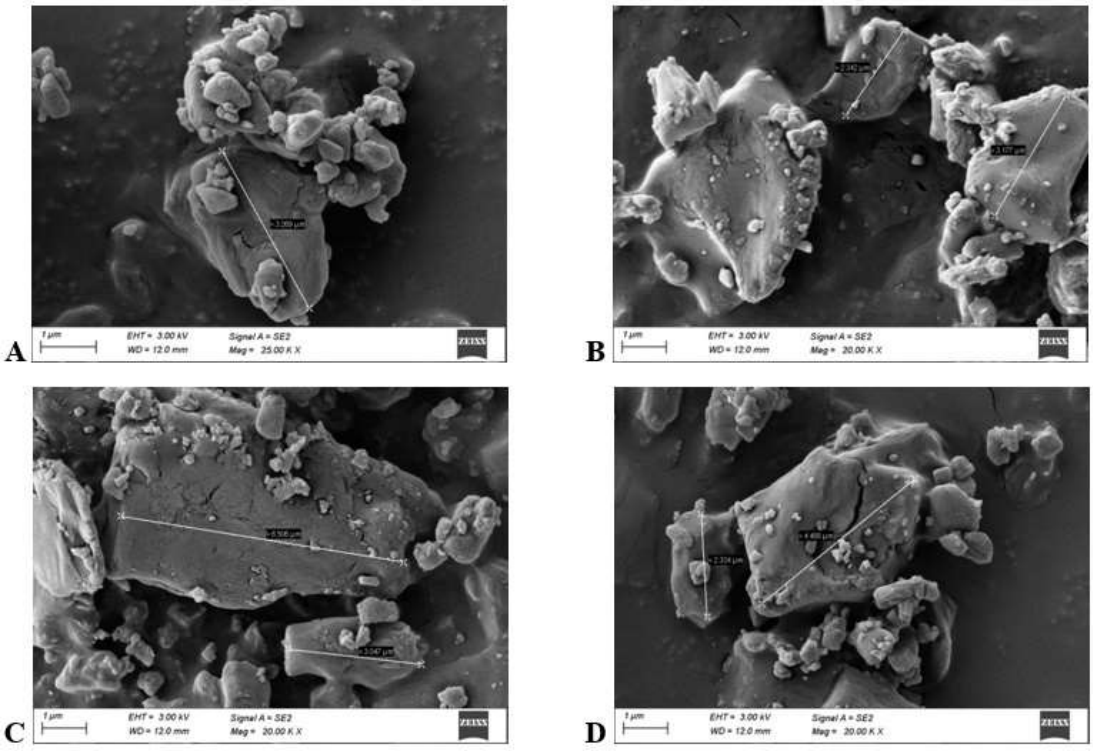

Fig. 2 SEM of developed formulation A F9, B F10, C F11 and D F12

An in vitro Release study was carried out using the dialysis sac method for up to 8 h, and a higher release was observed with the F12 formulation (86.18 %) followed by F11 (76.16 %) (Table-4).

Table 4 Results of In-Vitro Drug Release

TIME (h)	F9 (%)	F10 (%)	F11 (%)	F12 (%)
0.5	24.68	26.16	28.93	33.08
1	37.19	40.85	36.25	48.56
2	45.32	57.59	60.18	67.46
4	64.28	69.51	71.19	78.29
8	70.95	72.87	76.16	86.18

Cytotoxicity study:

Table 5, showed per cent cell viability values and observed IC_{50} value of Docetaxel and F12 against A549 cells after the treatment period of 24 hrs.

Table 5 % cell viability values and observed IC50 value of Docetaxel& F12 against A549 cells after the treatment period of 24hrs

Culture condition	Untreated	Docetaxel µM					F12				
		6.25	12.5	25	50	100	6.25	12.5	25	50	100
% cell viability	100.00	71.01	66.24	55.02	45.94	36.04	87.06	80.30	69.57	50.38	2.04
IC_{50} concentration (µM/ml)		36.28 µM/mL					47.54 µM/mL				

Drug-excipient compatibility studies using FTIR

The FTIR spectra of the individual components, including Docetaxel, cholesterol, and vitamin E TPGS, were analyzed separately. Additionally, a combination of the FTIR spectra was graphically represented to assess the compatibility between the drug and excipients, specifically Docetaxel with Cholesterol and Docetaxel with Vitamin E TPGS. The molecular structure of DTX represented N-H stretching, C=O stretching, O-H bending, and C-O stretching with absorption spectrum of 3550-3200 cm^{-1}, 1870-1705 cm^{-1}, 1440-1310 cm^{-1}& 1310-1020 cm^{-1} respectively. Based on the absorption observed under the wavelength, no interaction was observed between the drug and the polymer used (Table 2).

Moisture Content:

For the lyophilized product of the dry powder inhaler for the pulmonary route of drug delivery, the acceptable moisture content value is NMT 2.0% for greater stability, less degradation, and flow hindrance of the product [45,46]. Sartorius MA160 was used for the analysis, which involved measuring the moisture content of each formulation that was developed. All formulations had moisture contents below 2.0%, which is considered an excellent parameter to choose the pulmonary route of drug delivery.

Angle of Repose:

A decrease in the particle size exhibits flow properties owing to an increase in the surface area, and as a result, aerosolization performance within the inhaler exhibits a flow property [47, 48]. The angle of repose of formulations F1 and F2 was between 31 and 40, which is a passable flow property. Formulations F3 to F8 were found to be between 26 and 30, which indicates good flow properties, and formulations F9 to F12 were found to be less than 25, which indicates excellent flow properties. The flow property angle of repose varied owing to the irregularly shaped particles and moisture content. It can be inferred that formulations F9, F10, F11, and F12 exhibit superior flow properties.

Drug Content & Encapsulation Efficiency

All the developed formulations had a drug content of 95–105%[49,50,51]. When the encapsulation efficiency of each formulation was examined, it was discovered that the F1 formulation had an encapsulation of less than 30%. Due to a minor increase in the content of the cholesterol and vitamin TGPS polymer, formulations F2 and F3 had an encapsulation of 31% to 40%. Encapsulation ranged from 41% to 70% for formulations F4 to F7, with additional increases in the concentration of cholesterol and vitamin TGPS, and more than 71% for formulations F8 to F12. Furthermore, high encapsulation efficiencies of 75.91%, 77.15%, 81.25%, and 89.18% were achieved by the formulations of F9, F10, F11, and F12, respectively.

Regression models

Based on the JMP Software the DOE was performed to check the regression models of the developed formulation using factors and responses. Below are the tabulated data predicated from DOE software and the data predicted that the R^2 value is higher to 1 and RMSE value is near to 0. Response Angle of Repose of R Square & Root Mean Square Error was found to 0.9362 & 1.0539 respectively. Response Encapsulation Efficiency of R Square & Root Mean Square Error was found to 0.9754 & 4.1898 respectively. Based on the data available the developed formulation fit linear regression mode.

Scanning Electron Microscopy

F9, F10, F11, and F12 were chosen from among the 12 formulations based on their encapsulation efficacy of greater than 75%. Every formulation was analyzed at a distinct 20.00 K magnifications. The largest docetaxel formulation particle size, exhibiting spherical and quasi-spherical morphology, was ≤ 6 nm, as shown in figure-2.

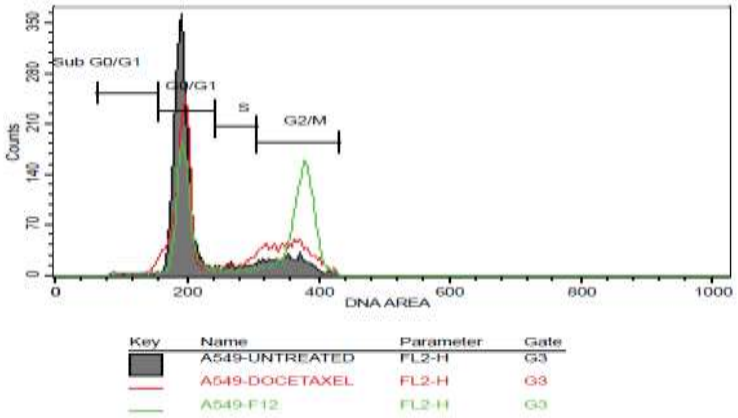

Fig. 3 Overlaid histogram of cell cycle distribution in the A549 cell line

In vitro Release – Dialysis Sacs Method

The release of the drug from the polymer varied according to the type and proportion of excipients. The in vitro drug dissolution profile of the polymeric particle showed that 45.32%, 57.59%, 60.18%, and 67.46% of the drug was released till 2 h from F9, F10, F11, and F12 formulations, respectively. From 2 to 8 h, the marked percentage releases were found to be 70.95%, 72.87%, 76.16%, and 86.18% from the F9, F10, F11, and F12 formulations, respectively. Formulation F12 exhibited the highest drug release (86.18 %) at the end of 8 h.

Cytotoxicity study by MTT Assay

With low IC_{50} values of 36.28µM/ml and 47.54µM/ml, respectively, docetaxel and F12 were found to be efficaciously cytotoxic against human alveolar lung cancer (A549) cells, according to the results of a cytotoxicity study conducted using the MTT test.

Cell cycle analysis of A549 cells by flow cytometry

In Table 6, comparison studies were performed between the untreated cells, Pure Docetaxel API, and the developed formulation with IC_{50} concentrations. The IC_{50} concentration of

Docetaxel API and F12 was 36.28 µM/ml & 47.54 µM/ml respectively was used. It was found that the G0/G1 phase (apoptotic phase), 0.58%, 2.32%, and 0.79% cells were inhibited in untreated, Docetaxel and F12, respectively. G0/G1 phase (Growth Phase): 77.76%, 56.39%, and 41.11% cells were arrested in Untreated, Docetaxel and F12, respectively. S phase (synthetic phase) 5.47%, 7.96%, and 3.41% of cells were arrested in Untreated, Docetaxel and F12 cells, respectively, whereas in the G2/M phase, 16.19%, 33.33%, and 54.69% of cells were arrested in untreated, docetaxel and F12 with IC_{50} concentrations respectively. It was concluded that the developed F12 caused effective cell cycle distribution in A549 cells at G2/M phase compared to docetaxel, did not allow the cells to undergo cell division, and was confirmed to be a potent anti-lung cancer drug.

Table 6 % cells get arrested in the different phases of A549 cell cycle

Cell Cycle Study-A549			
Cell Cycle stage	**Untreated**	**Docetaxel**	**F12**
Sub G0/G1	0.58	2.32	0.79
G0/G1	77.76	56.39	41.11
S	5.47	7.96	3.41
G2/M	16.19	33.33	54.69

4. CONCLUSION:

Docetaxel remains a potent anticancer drug that faces a challenge in the delivery of drugs; the commercially available drug is intervenors only, and patient compliance can also be improved when a Dry Powder Inhaler is provided, which affects only the lungs rather than indicating intravenous, which affects all parts of the erecting organ. Attempts have been made to develop a dry Powder Inhaler for Docetaxel formulation using Cholesterol and Vitamin TGPS to increase the efficacy and efficiency of drug delivery. Based on these studies, it was evident that the flow properties of the developed formulation were excellent, and in vitro studies showed sustained release profiles, with formulation F12 exhibiting the highest release (86.18%) at 8 h. Through MTT assay on A549 cell lines for IC_{50} of pure API and F12 have 36.28µM/ml and 47.54µM/ml respectively and in cell cycle analysis conclude that the developed F12 caused effective cell cycle distribution in A549 cells at G2/M phase than the Docetaxel and won't allow the cells to undergo cell division and confirmed to be potent anti-lung cancer drug. Further detailed investigations are required to establish the clinical efficacy of the developed formulation of Dry powder Inhaler.

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CONFLICT OF INTEREST

None reported

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histogram of cell cycle distribution in the A549 cell line

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