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PAMAM G4 Dendrimers & Vitamin D Nano Complex for delivery of Doxorubicin

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Abstract- In cancer treatment, efficient drug delivery systems are imperative to enhance therapeutic efficacy while minimizing systemic toxicity. This study explores the potential of a novel nano-complex composed of PAMAM G4 dendrimers and vitamin D for the targeted delivery of doxorubicin (DOX), a widely used chemotherapeutic agent. The synthesis and characterization of the nano-complex were meticulously conducted, elucidating its physicochemical properties and structural integrity.The encapsulation of DOX within the nano-complex was optimized, ensuring high drug loading efficiency and sustained release kinetics. Moreover, the synergistic effects of vitamin D were harnessed to augment the anticancer activity of DOX while mitigating its adverse effects on normal tissues. Cellular uptake studies revealed enhanced internalization of the nano-complex in cancer cells, facilitated by receptor-mediated endocytosis mechanisms.In vitro studies demonstrated the superior cytotoxicity of the DOX-loaded nano-complex compared to free DOX, attributed to its targeted delivery and intracellular release. Furthermore, the nano-complex exhibited favorable biocompatibility profiles, minimizing off-target effects on healthy cells. In vivo efficacy assessments using xenograft tumor models corroborated the enhanced therapeutic outcomes of the nano-complex, showcasing significant tumor growth inhibition and prolonged survival rates compared to conventional DOX formulations. Pharmacokinetic studies indicated improved circulation half-life and bioavailability of DOX when delivered via the nano-complex. Overall, the developed PAMAM G4 dendrimer and vitamin D nano-complex represent a promising strategy for the targeted delivery of DOX in cancer therapy, offering enhanced therapeutic efficacy, reduced systemic toxicity, and improved patient outcomes. Further optimization and translational studies are warranted to validate its clinical potential and pave the way for its implementation in cancer treatment regimens.

KEYWORDS- Dendrimer, Doxorubicin, Vitamin D, Cancer therapy, Drug release kinetics, Targeted delivery, In vivo efficacy.

INTRODUCTION

Cancer is the abnormal growth of cells. Cancers arise from any organ or body structure and are composed of tiny cells that have lost the ability to stop growing. Occasionally, cancer may be detected ″incidentally” by a laboratory test or radiological routine test or for an entirely different reason. In general, cancer must reach a size of 1 cm, or be comprised of 1 million cells, before it is detected. At this point, it may be referred to as a ″'mass,” a ″growth,” a ″tumor,” a ″nodule,” a ″lump,” or a ″lesion.” Exceptions to this general rule include cancers of the blood and bone marrow (leukemia's and lymphomas) – which frequently do not produce a ″mass,” but will be evident on laboratory tests.

Transformation of a normal cell into a cancerous cell is probably not such a critical event in the genesis of cancer; rather it is the inability of immune cells of the body to identify and destroy the newly formed cancer cells when they are a few in numbers[1].

EPIDEMIOLOGY

According to estimates from the ″International Agency for Research on Cancer (GLOBOCAN 2012), there were 14.1 million new cancer cases, 8.2 million cancer deaths, and 32.6 million individuals living with cancer in the world in 2012". Economically developing countries are predicted to account for 57 percent (8 million) of new cancer cases, 65 percent (5.3 million) of cancer fatalities, and 48 percent (15.6 million) of cancer cases. At the same time, India's overall cancer population was estimated to be at 1.2 billion people, with 0.9 million new cancer diagnoses and 0.6 million cancer deaths each year. Despite this, cancer incidence is anticipated to rise to around 27 million new cases and 17.5 million cancer deaths by 2050, owing to aging, population increase, and the adoption of cancer-causing behaviors, as well as the limitations of cancer treatment[2,3,4].

DENDRIMERS

Dendrimers are nano-sized, radially symmetric molecules with well-defined, homogeneous, and monodisperse structure consisting of tree-like arms or branches[5]. These hyperbranched molecules were first discovered by Fritz Vogtle in 1978, by Donald Tomalia and co-workers in the early 1980s, and at the same time, but independently by George R Newkom. Dendrimers are sometimes known as "cascade molecules," however this name is less well-known than "dendrimers"[6].

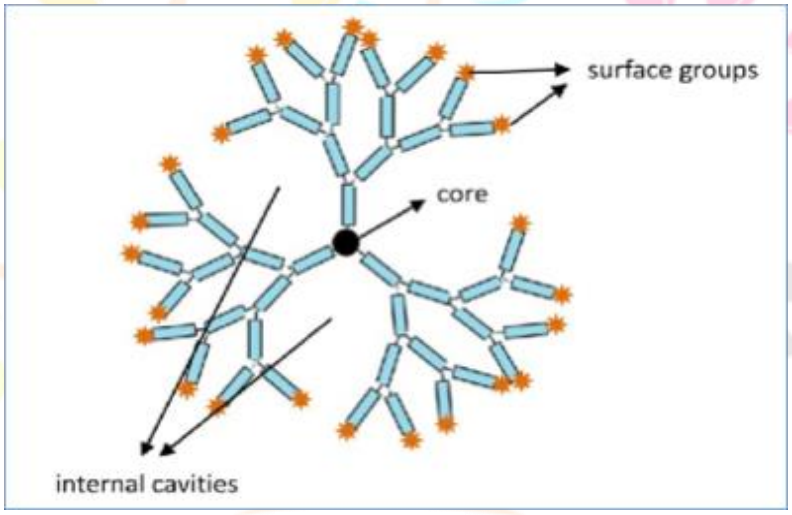
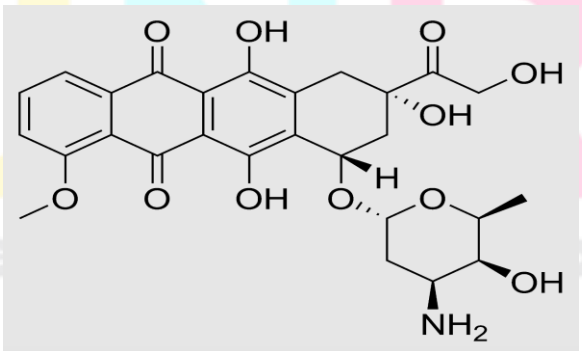


Fig 1. Structure of dendrimer

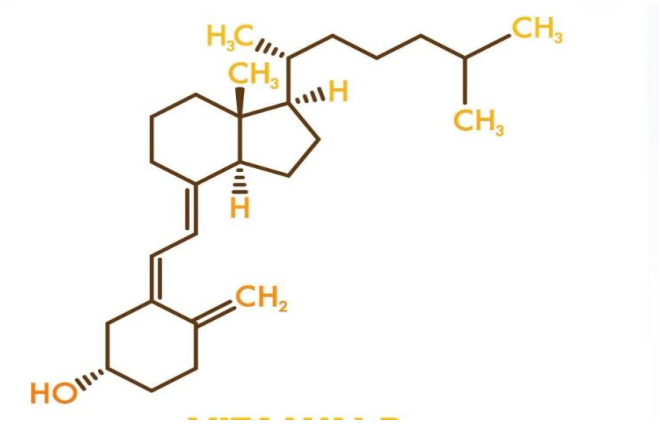


DOXORUBICIN

Fig 2. Structure of Doxorubicin

Doxorubicin is an anthracycline antibiotic that has anticancer properties. Doxorubicin is the hydroxylated congener of daunorubicin, which was obtained from the bacterium *Streptomyces peucetius* var. caesius Doxorubicin binds to base pairs in the DNA helix, limiting DNA replication and, as a result, reducing protein synthesis. Doxorubicin is a drug that is used to treat cancers that have spread throughout the body like breast carcinoma, acute myeloblastic leukemia, Hodgkin′s disease, ovarian carcinoma, gastric carcinoma,

neuroblastoma, thyroid carcinoma, soft tissue and bone sarcomas, acute lymphoblastic leukemia, malignant lymphoma[7].



VITAMIN D

Fig 3. Structure of Vitamin D

Vitamin D is a group of fat-soluble hormone and prohormones. Vitamin D has been found to have several biological activities that might slow or prevent the development of cancer, including promoting cellular differentiation, decreasing cancer cell growth, stimulating cell death (apoptosis), reducing tumor blood vessel formation(angiogenesis) and decreasing tumor progression and metastasis. Vitamin D was also found to suppress a type of immune cell that normally prevents the immune system from responding strongly to cancer[8].

MATERIAL AND METHOD

PREFORMULATION STUDY

The development of sensible dosage forms of a pharmacological substance begins with pre-formulation testing.

Solubility Analysis

A drug and excipients were dissolved in a 10 ml buffer solution separately with pH values of 6.8 and 7.4 in a conical flask and shaken for 24 hours with the help of a conical flask shaker and were then filtered with Whatman filter paper No. 1, diluted accordingly and UV spectroscopy was used to examine it.

Melting Point Determination

The melting point of the drug and excipients were determined by a digital melting point apparatus.

Partition Coefficient Measurement

The drug and excipients were dissolved in a volume of octane and water by shake-flask method and then the concentration of solute in each solvent was determined by a UV spectrophotometer by which logP can be determined.

Loss on Drying

An empty Petri dish was washed, dried, and precisely weighed. The Petri dish was filled with around 1.0 grams of drug weighed and kept in a hot air oven at 105°C for 4 hours. It was weighed again, and the loss on drying was calculated using the formula below [9].

% Loss on drying = Weight of drug before drying - Weight of drug after drying / Weight of drug before drying ×100

Standard Calibration Curve

The standard calibration curve for doxorubicin was created and calculated using a UV-visible spectrophotometer at 484 nm by using phosphate buffer pH 7.4.

Preparation of Phosphate Buffer pH 7.4

800 mL of distilled water was taken in a suitable container, 8 g of NaCl and 200mg of KCl, 1.44g of

Na2HPO4 and 245mg of KH2PO4 were added to it. The volume was made up to 1 liter and the pH was adjusted to 7.4.

Procedure for the preparation of calibration curve by UV:
A standard drug stock solution (1 mg/ml) was prepared, and dilutions were made with a phosphate buffer pH 7.4 in 10 ml volumetric flasks. Using a UV-visible spectrophotometer, the absorbance of these solutions was measured at their respective wavelengths of maximum absorbance.

Fourier Transfer Infrared (FTIR):
The dendrimers were taken and mixed with about 100mg of KBr; the mixture was triturated and put into a cavity for compression. The formed disc was then subjected to FTIR examination which was analyzed within the range of 5000cm-1 to 500cm-1[10].

FORMULATION OF DOXORUBICIN G4 PAMAM DENDRIMER

40 mg of G4 PAMAM and their unconjugated native counterparts were dissolved in 4 ml of deionized water. In a round-bottom flask approximately 28.2 mg (equimolar 32 times of dendrimer) of doxorubicin was dissolved in 15 ml of deionized water, when the doxorubicin was completely dissolved, dendrimer solution was added to doxorubicin solution drop wise under stirring at room temperature. The solution was left to react for 4 h and dialyzed (3.5 kDa MWcut-off) against deionized water for 24 h[11].

TABLE 1. Formulation Development of Doxorubicin G4 PAMAM Dendrimer

S.NO.	FORMULATION	DENDRIMER (µl)	DOXORUBICIN (µl)	VITAMIN D (µl)	DEIONISED WATER (ml)
1	DPF1	70	2240	560	15
2	DPF2	70	2240	1120	15
3	DPF3	70	2240	2240	15
4	DPF4	70	2240	3360	15
5	DPF5	70	2240	4480	15

Physical Characterization of Dendrimer Formulations
The dendrimer formulations were evaluated by following properties like particle size analysis, polydispersity index and zeta potential analysis.

Determination of Particle Size Distribution
The average vesicle size of the doxorubicin dendrimer with carrier was a crucial factor to determine. It was carried out at the Central Drug Research Institute (CDRI) in Lucknow, utilizing Malvern Instruments.

Polydispersity Index
The electro-kinetic potential of liquid-liquid or solid-liquid colloidal dispersions is characterized using the zeta potential. Electrophoresis, streaming potential, electro-osmosis, and sedimentation potential are all electro-kinetic effects that charged particles display when exposed to an electric field.

Zeta Potential Analysis

The value of the ″zeta potential is related to the stability of colloidal dispersions, which is why it is so important colloids with a high zeta potential (negative or positive) are electrically stable, whereas those with a low zeta potential coagulate or flocculate”. The arbitrary value that distinguishes low-charged surfaces from high-charged surfaces is 25mV (positive or negative). Malvern Zetasizer at Central Drug Research Institute, Lucknow, analyzed the zeta potential.

Drug Content

To obtain the perfect solubility of the drug, a particular quantity of the produced formulation was dissolved in 100 ml of methanol and agitated for 2 hours on a mechanical stirrer. This solution was filtered through a Millipore filter (0.45 m), and drug absorption was measured using a UV spectrophotometer with methanol as a blank.

Entrapment Efficiency

Entrapment efficiency of formulations was found by indirect method. 3ml of the formulation was taken and kept in a quartz silica cell. Blank was used as an HPLC water. The absorbance of formulations was taken at 386nm by UV-VIS spectroscopy. This equation was used to calculate entrapment efficiency[12]:

Entrapment Efficiency = Initial weight – Free weight Initial weight × 100

pH Measurement

pH measurement was performed by using a calibrated pH meter. The standard calibration pH solutions of 4.1, 7.0, and 9.0 on further the solution were measured.

In vitro Drug release studies

4 ml of dendrimer suspension with a known amount of medication was placed in a dialysis membrane that had been soaked overnight for in-vitro drug release from the dendrimer formulations. The two-sided open cylinder was submerged in 200 mL PBS (pH 7.4), kept at 37 °C, and agitated with a magnetic stirrer. To maintain a consistent volume, aliquots (4 ml) of release medium were withdrawn at varied time intervals and replenished with fresh PBS (pH 7.4). To precipitate the lipids and dissolve the entrapped doxorubicin, 1 ml acetonitrile was added to each aliquot, and the samples were then examined with a UV spectrophotometer at a λmax of 284 nm.

Short-Term Stability Studies

The capability of a certain formulation in a specific container can be defined as the stability of a pharmaceutical delivery system. The short-term stability study was carried out to test the physical and chemical stability of the liquid form of doxorubicin dendrimer formulations for up to three months at 40°C and room temperature. As a function of storage time, stability parameters such as particle size and zeta potential were assessed [13].

RESULTS AND DISCUSSION

Pre-formulation Studies

Doxorubicin was procured by Tristar Formulation Pvt. Ltd., Puducherry. It was identified and characterized as per the identification test given in the Indian Pharmacopoeia (2010) and United State Pharmacopoeia.

Identification of the Drug

I) Physical Property

Colour: Red
Odor: Odorless
Taste: Bitter
State: Crystalline powder

Solubility Study of the Drug

Doxorubicin was Soluble 1 in 20 of water, 1 in 5 of ethanol, 1 in 2 of chloroform, and very slightly soluble in ether. The solubility analysis of drug was performed acc. to the procedure and result is shown in following table:

TABLE 2. Solubility analysis of Doxorubicin

S.NO.	DRUG	SOLVENT			
		DISTILLED WATER	0.1N HCl	PHOSPHATE BUFFER pH 6.8	PHOSPHATE BUFFER pH 7.4
1	Doxorubicin	0.0156 g/ml	0.0167 g/ml	0.0156 g/ml	0.0065 g/ml

Melting Point Determination

The melting point of the drug sample was discovered to be 215.6 °C using the procedure described in the materials and methods section. The drug's melting point is said to be between 216 and 230 degrees Celsius. As a result, the drug sample meets USP requirements, showing its purity.

Partition coefficient of Doxorubicin

The drug's partition coefficient was determined using the procedure outlined in the materials and methods section. The average log P value was discovered to be between 1.28 and 1.29. The drug's log P standard value is 1.27.

TABLE 3. Partition coefficient of Doxorubicin

Water: n-octanol (ml)	Conc. Of drug in water (µg/ml)	Conc. of drug in n-octanol (µg/ml)	Log P	Reference value of Log P
1:1	6,56	21,87	1,28	1,27
1:1	6,89	22,84	1,19	

Loss on Drying

LOD of the sample drug was performed acc. to the procedure mention in materials and methods. According to USP monograph of Doxorubicin, % loss of the drug should not be more than 1% of its weight and the result of LOD of Doxorubicin is shown in table 3.4. The average LOD of Doxorubicin was found to be 0.52, Hence it will complies with the standards.

TABLE 4. %Loss on drying of Doxorubicin(50g)

S. No.	Wt. of drug before drying (mg)	Wt. of drug after drying (mg)	LOD (%w/w)	Average LOD (%w/w)
1	50	44,7	0,53	0,52
2	50	43.8	0,49	

Standard Calibration Curve of Doxorubicin hydrochloride in UV spectrophotometer

UV absorbances of doxorubicin hydrochloride standard solution in buffer pH 7.4 demonstrated linearity at max 484 nm in the range of 10-50 g/ml of drug. The R^2 value of 0.9998 and the slop equation y=0.179x-0.003 were used to plot the linearity of absorbance against concentration. Table 3.4 shows the absorbance readings as well as a standard curve.

Table 5. Standard Reading of Doxorubicin in UV spectrophotometer

Concentration (µg/ml)	Absorbance at 484 nm
0	0
10	0,181
20	0,342
30	0,531
40	0,719
50	0,899

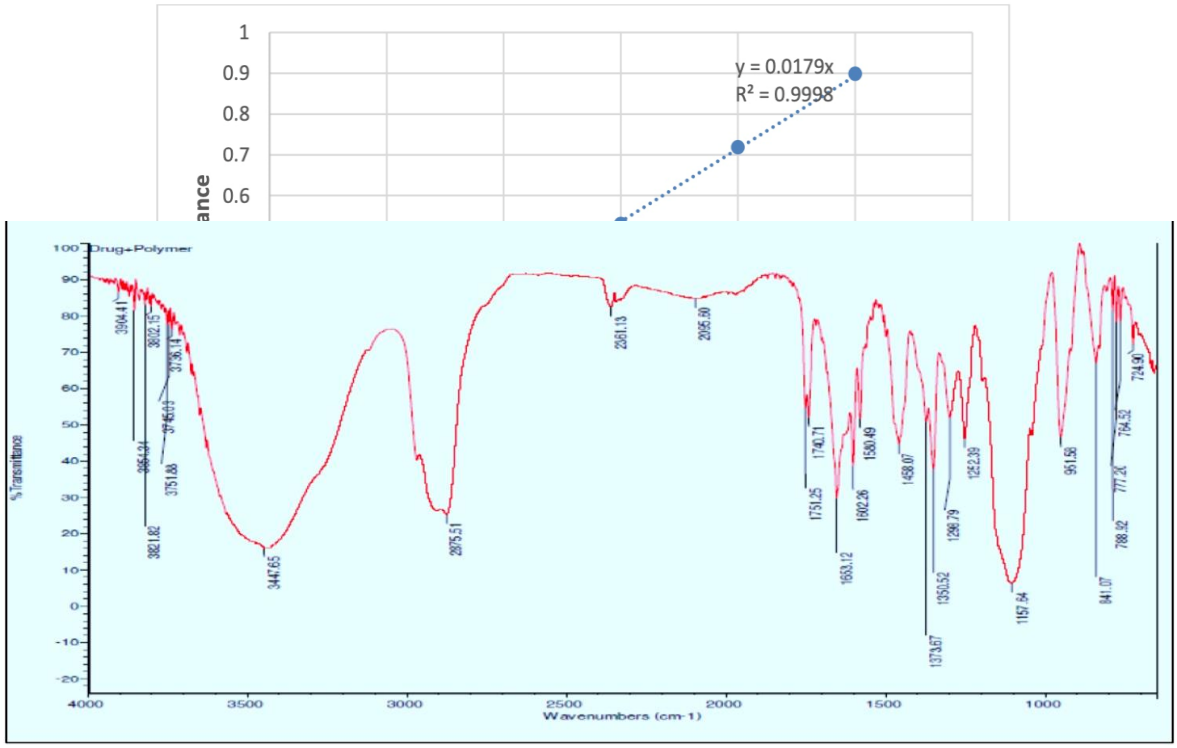


Fig 4. Calibration curve of Doxorubicin

The FTIR peak matching approach was used to determine the drug's compatibility with a number of additional excipients. Peaks in the drug-excipient mixture did not emerge or vanish, indicating that there was no chemical interaction between the drug and the other substances. Table below displays the results.

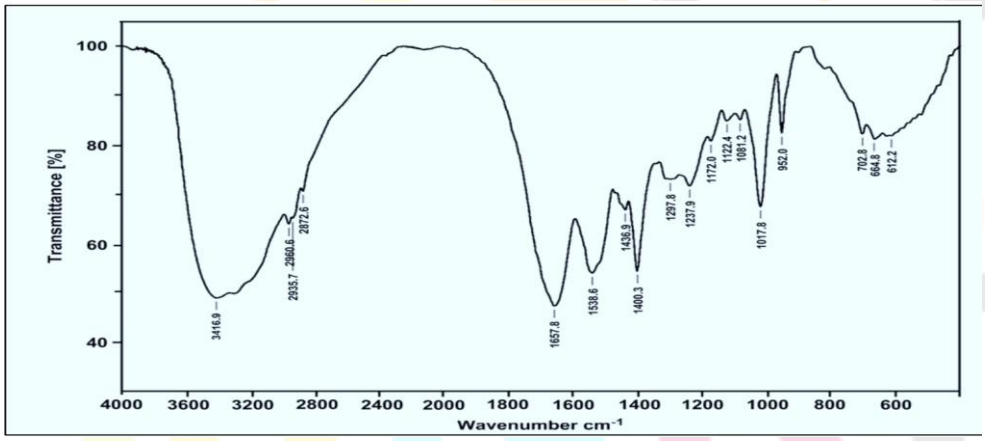


Fig 5. FTIR Spectra of pure drug doxorubicin

Table 6. Interpretation of FTIR Spectra doxorubicin

S. No.	Assessment Peak of Drug	Functional group
1	1524,51	C=C Stretching (Aromatic)
2	1072,51	O-H Bending (Alcohol)
3	1729,84	C=O Stretching
4	1617,83	N-H Bending
5	1114,46	C-O Stretching

Fig 6. FTIR Spectra of formulation

Table 7. Interpretation of FTIR Spectra (Dendrimer Formulation)

S. No.	Assessment Peak of Drug	Functional group
1	1524,51	C=C Stretching (Aromatic)
2	1072,51	O-H Bending (Alcohol)
3	1729,84	C=O Stretching
4	1617,83	N-H Bending
5	1114,46	C-O Stretching

Formulation Characterization

Particle Size Distribution

For the DPF1, DPF3, and DPF4 doxorubicin dendrimer formulations, the particle size distribution was investigated. Table 3.7 shows that the particle size in DPF1 formulation was optimal when compared to DPF3 and DPF4.

Zeta Potential Analysis

DPF1, DPF3, and DPF4 dendrimer formulations have zeta potentials of -13.1mV, 4.66mV, and -15.8 mV, respectively, which are close to the arbitrary value. The findings demonstrate that the formed solution has a high level of stability; the results are presented in table.

Table 8. Physicochemical characteristics of doxorubicin dendrimer

S. No.	Formulation code	Average vesicular size (nm)	Zeta Potential (mV)	Poly dispersive index (PDI)
1	DPF1	183.1 nm	-13,1	0,132
2	DPF3	198.6 nm	4,66	0,156
3	DPF4	201.7nm	-15,8	0,167

Fig 7. Particle size distribution of formulation DPF1

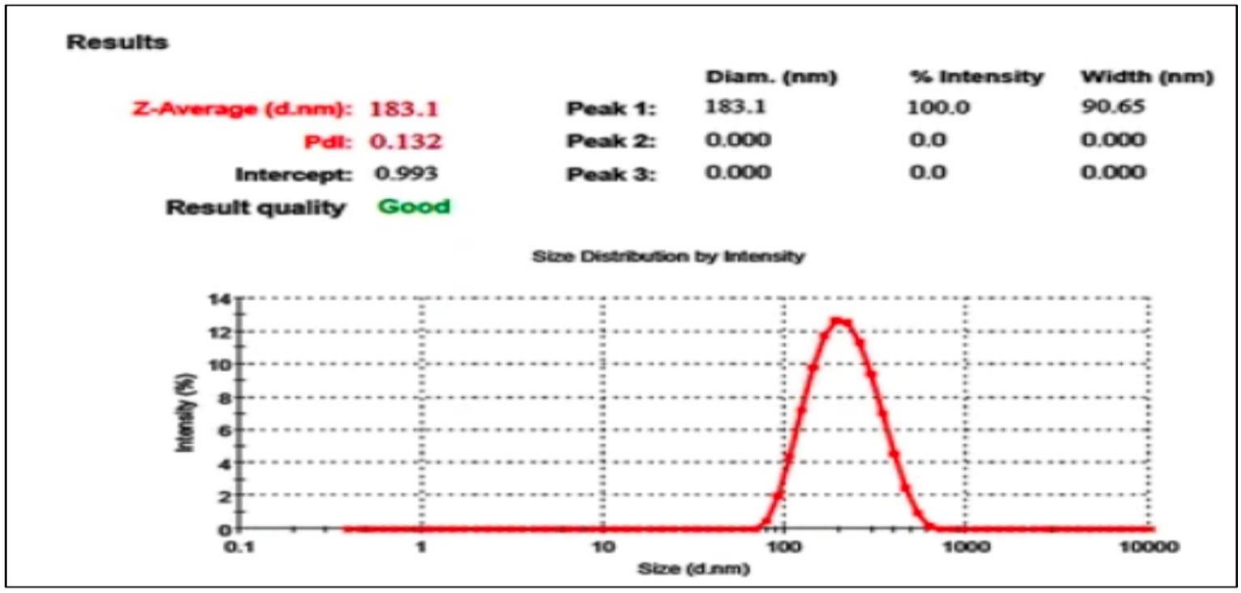
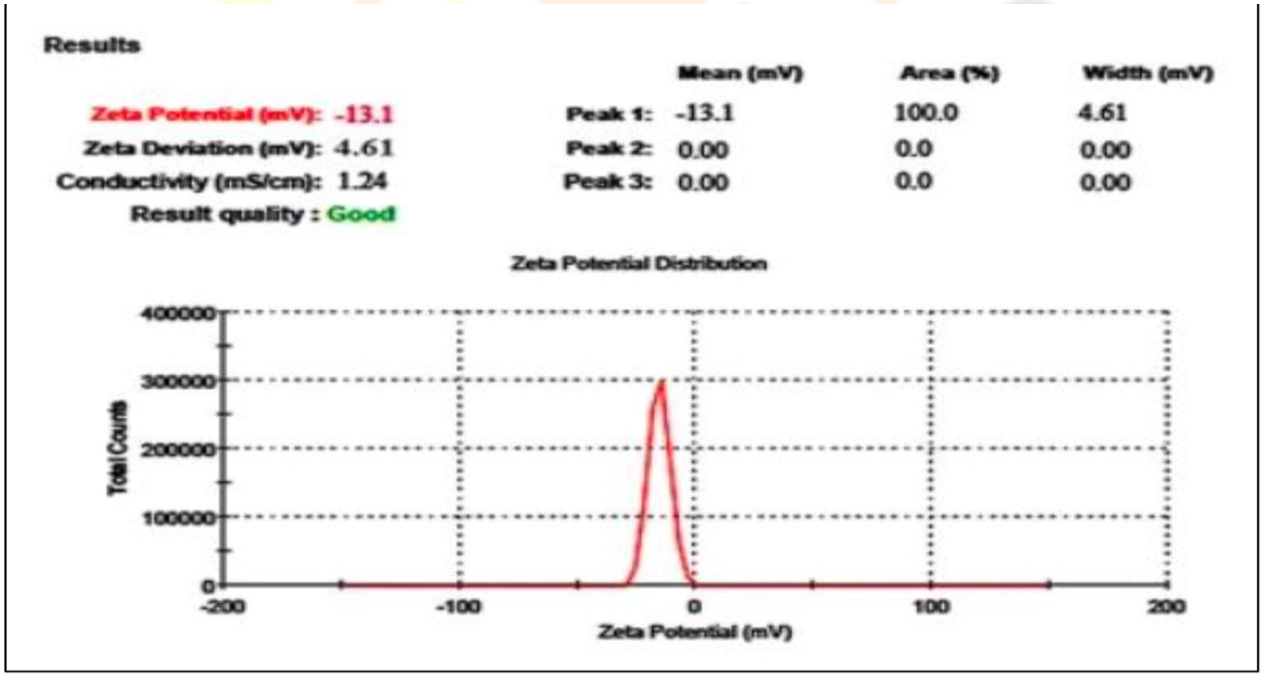


Fig 8. Zeta Potential of formulation DPF1

Drug Content Analysis

The mean percent drug content in dendrimer formulations (DPF1-DPF5) was found to be respectively (92.99%, 89.65%, 90.54%, 88.99% and 90.32%).

Table 9. Drug Content Analysis of Dendrimer Formulations



Formulation	% Drug Content
DPF1	92,99
DPF2	89,65
DPF3	90,54

DPF4	88,99
DPF5	90,32

Entrapment Efficiency

The trapping efficacy of the dendrimer formulations was calculated by the process of direct method. DPF1 demonstrated maximum entrapment efficiency as shown in table.

Table 10. Entrapment Efficiency of Dendrimer Formulations

Formulation	Entrapment Efficiency
DPF1	59,93 %
DPF2	54,21 %
DPF3	49,65 %
DPF4	50,91 %
DPF5	44,34 %

pH

In the range of 6.09 to 6.76, the pH of the formulations avoid the possibility of irritation. In table the result is shown. It was noticed that the optimised formulation DPF1 pH was 6.54.

Table 11. pH of Dendrimer Formulations

Formulation	pH
DPF1	6,37
DPF2	6,43
DPF3	6,34
DPF4	6,49
DPF5	6,98

In-Vitro Drug Release

In vitro drug release studies from DPF1-DPF5 batch different dendrimer formulation, all having the same quantity. This was done to maintain the sink condition. In vitro drug release were the highest in formulation DPFQ. The significant difference in Dendrimer formulation was probably due to the mean size of vesicles. Prepared formulation showed a drug release profile over 24 h. In vitro drug release indicates how a drug will behave in- vivo.

Table 12. *In vitro* cumulative % drug release profile of doxorubicin Dendrimer formulations

TIME (Hrs)	Cumulative % drug				
	DPF1	DPF2	DPF3	DPF4	DPF5
1	3,12	2,01	1,99	2,87	2,33
2	7,76	3,99	3,88	4,37	5,45
4	14,67	8,89	6,99	7,11	8,98

6	19,98	14,63	14,09	16,21	17,92
8	27,21	21,65	19,86	24,87	22,21
12	39,09	29,91	26,95	31,45	28,35
16	47,89	39,73	35,62	39,82	35,95
20	63,85	49,25	41,56	47,78	47,86
24	78,85	61,31	59,59	63,84	67,25

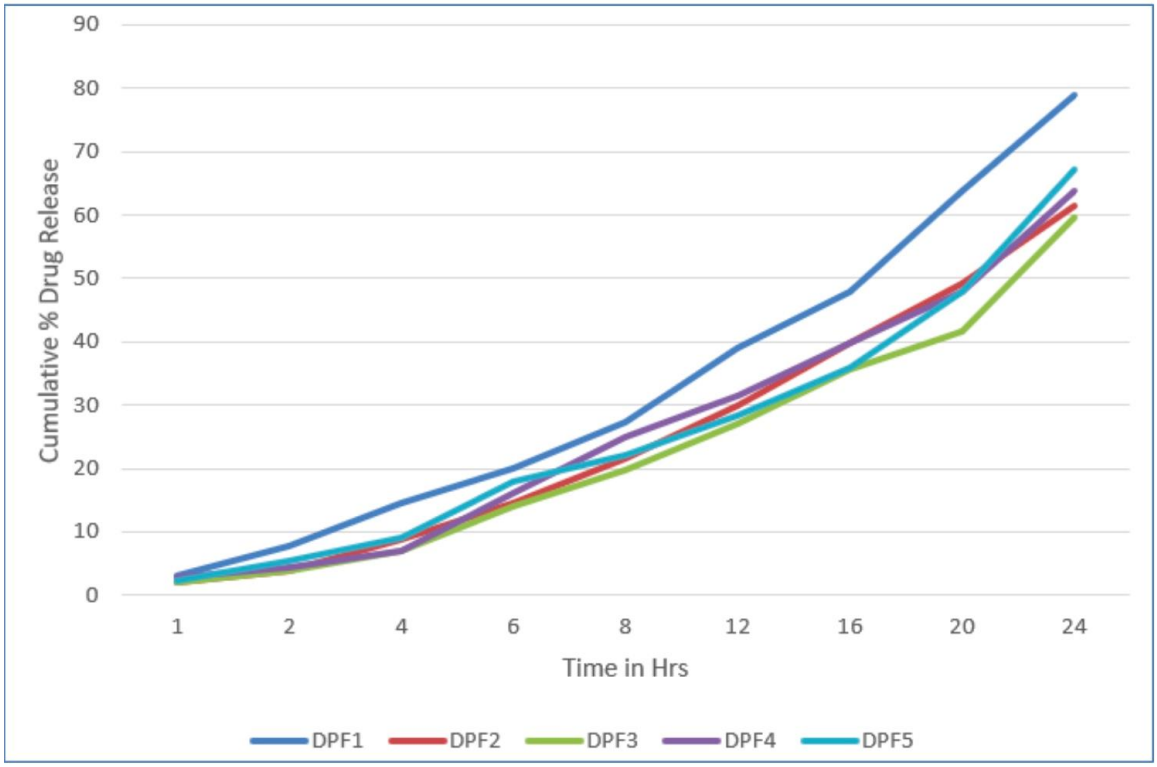


Fig 9. Cumulative % Drug Release

Stability Data

The doxorubicin dendrimer's stability was tested for 60 days at 4 degrees Celsius and room temperature. As a function of storage time, the sample assays were determined. The dendrimer was found to be stable for 60 days when kept at 4 degrees Celsius. Table given below summarizes the findings.

Table 13. Stability study of Dendrimer formulations

Characterization	**DPF1**			
	0 MONTH	2 MONTHS	4 MONTHS	6 MONTHS
Particle size (nm)	183.1nm	182.99 nm	182.81 nm	182.57 nm
PDI	0,132	0,132	0,131	0,131
Zeta potential	-13,1	-13,4	-13,13	-13,14

CONCLUSION

The primary goal of this study was to create and test a Doxorubicin dendrimer formulation including Vitamin D. The influence of various stabilizers on drug entrapment efficiency will be used in this formulation to target the site of action, and dendrimer will be used to decrease adverse effects. The vitamin used in the dendrimer formulation is less harmful.

After 60 days of storage at 4 ℃ and room temperature, the stability of the Doxorubicin dendrimer was determined. The assay of the samples was calculated as a function of storage time. The Dendrimer was found to be stable over a three-month period when stored at 4 ℃.
Physical characterization, in-vitro evaluation, in-vitro drug release, and stability studies are all based on the results of the physical characterization.

″Even while preliminary data based on in-vitro dissolution profiles, release kinetics, and stability tests showed that such formulations are suitable, animal studies will require a more extensive investigation”. Following that, we can determine the dosage form's true method of action.