



## **Tissue Distribution, Cytotoxicity and Pharmacokinetic Studies of Multifunctional Citric Acid Dendrimers using the Drug Cyclophosphamide**

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**Abstract :** The aim of present work is to develop a multifunctional dendritic carrier for targeting tumor cells that overcome drug leakage during circulation and encapsulate tightly an anticancer drug within it through high affinity interactions. PEGylation, polyethylene glycol addition to dendritic carriers improves its circulation time in the body and also ensures high stability without detectable drug release from formulations other than the target site. PEGylated citric acid dendrimer was found an efficient carrier for targeting antitumor agent, cyclophosphamide to the target tumor organs. Dendrimers are repeatedly branched, spherical molecular moieties synthesized by Divergent technique and characterized for acute cytotoxicity assays, pharmacokinetic parameters and tissue distribution studies. Results of the above studies met the objective and tumor uptake of cyclophosphamide has increased significantly when compared with other organs and exhibited relatively lower toxicity to other organs targeting specifically tumor cells. Thus, the data suggest PEGylated citric acid dendrimer-drug complex is a simple and efficient carrier system to deliver drugs to the tumor cells.

**Key words :** Dendrimer, Acute toxicity, Tumor, myeloid, melanoma, multi-functional.

### **Introduction:**

Melanoma and Leukemia are considered the leading causes of cancer deaths world-wide<sup>1</sup>. Melanoma, a skin cancer, increasing at a rate faster than any other cancers whereas myeloid leukemia, neoplastic proliferation of myeloid cells represents the maximum number of deaths<sup>2</sup>. Chemotherapy of the above cancers is associated with several other undesirable and unwanted side-effects. Therefore, there is a need to control the incidence of these cancers either by early detection or by employing proven prevention methods and techniques<sup>3</sup>. Many studies proved that nanotechnology creates a new pathway for some nano drug delivery systems that reduce the unwanted or undesirable side-effects produced during chemotherapy. In addition, it enhances the efficacy of the drug at the target site<sup>4</sup>. An ideal nanotechnology based tumor therapeutics should be a multifunctional complex device but small enough to leave the vasculature for its specific interaction with the cancer cells followed by their elimination from the body<sup>5</sup>.

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Citric acid dendrimers are repeatedly branched polymeric molecules with multiple functional end groups at its terminal surface that provide accessibility to encapsulate a variety of substances such as therapeutic agents, imaging agents, contrasting agents, targeting moieties etc<sup>6</sup>. Drugs either get encapsulated into the interior or conjugated to the surface of dendrimers<sup>7</sup>. Drug retention and their subsequent release from the host dendrimers is strongly affected by the strength of the drug-dendrimer interaction and the water solubility of dendrimers<sup>8</sup>. Properties such as well-defined structure with a monodispersed molecular weight makes them suitable for targeting molecular entities precisely to the cancer tissues through EPR effect<sup>9</sup>. Being water-soluble, citric acid dendrimers prolong the half-life of encapsulated drugs. They take advantage of EPR effect which is commonly observed with tumor cells. Blood vessels of tumors are poorly aligned and possess proliferations in its structure<sup>10</sup>. Owing to this property of tumor cells, nano sized dendrimers easily pass into them without disturbing other healthy cells. Because of poor lymphatic drainage, dendrimers accumulate in tumor cells for a longer time to release the encapsulated drug into them, which simultaneously exhibits therapeutic activity and stand responsible for its destruction<sup>11</sup>.

Cyclophosphamide, a nitrogen-mustard (alkylating agent) is effective against both leukemia and melanoma. CYC is considered among the most potent immunosuppressive drugs today. When administered into body, inactive CYC gets converted to active phosphoramidate and acrolein compounds. Formed phosphoramidate mustard forms DNA cross-linkage and interferes with the DNA replication. So that the cross-linked tumor cell cannot undergo normal cell division, causing the tumors cells to die<sup>12,13,14</sup>.

## Materials and Methods

**Materials :** Citric acid dendrimers- G5, Cyclophosphamide was purchased from Yarrow Chemicals, Mumbai; Polyethylene glycol 400 and chloroform were purchased from Finar Chemicals Pvt. Ltd., Ahmedabad; Acetonitrile and N, N'- Dicyclohexylcarbodiimide were bought from Sd Fine Chemicals Ltd., Mumbai. All the chemicals used were of good analytical grade.

**Animals :** B16F10 animals were procured from NIN, Hyderabad for their use in pharmacokinetic and biodistribution studies. All the animals were inbred for experimental purposes in accordance with CPCSEA guidelines. Animals were housed in individually ventilated cage system with a 12hr light/dark cycle. Noise, temperature and humidity were controlled as per the guidelines. They were supplied with standard rodent chow, water and bedding material before surgery. Statement of human and animal rights: This article contains studies with animal subjects performed by authors. Studies were performed after receiving approval from IAEC/CPCSEA. All institutional and national guidelines for the care and use of laboratory were followed. (IAEC/CPCSEA approval no.DL/IAEC/08/16/13).

**Cell lines :** B16F10 melanoma and K562 myeloid cell lines were procured from ATCC and cultured in DMEM supplemented with 10% FCS.

**Method :** Citric acid dendrimers were prepared, PEGylated followed by drug loading in the synthesized citric acid dendrimers.

**Synthesis and PEGylation of citric acid dendrimers :** 5<sup>th</sup> generation citric acid dendrimers were synthesized via the same divergent technique as reported in our previous work<sup>15</sup>. The synthesized dendrimers were PEGylated as per the following procedure: Synthesized dendrimers were dissolved in 10ml of DMF completely and 5 moles of N, N' dicyclohexylcarbodiimide was added and stirred thoroughly and kept aside undisturbed for 4 days. After 4 days, reaction mixture was esterified by adding 85ml of PEG and 150ml of chloroform to yield a clear solution. Resultant mixture was filtered through separating funnel by adding 5ml of water that removes completely the unreacted N, N'- dicyclo hexyl carbodiimide<sup>16</sup>.

**Drug loading in the PEGylated dendrimers :** Drug cyclophosphamide was dissolved in distilled water and added to the dendrimer solution dropwise and stirred for around 24hrs on a magnetic stirrer to ensure complete encapsulation of drug into dendrimers<sup>17</sup>.

**Characterization of PEGylated citric acid dendrimers :** Synthesized dendrimers were characterized for the following study parameters;

**MTT cytotoxicity assay :** MTT cytotoxicity assay was performed individually on B16F10 melanoma cells and K562 myeloid cells. Tumor cells were seeded in 96-well plates at a density of  $4.5 \times 10^3$  cells/well separately. Synthesized dendrimers were dissolved in DMSO and added to the above wells. Final concentration of citric acid dendrimers in wells was adjusted to get a concentration range of 0.001 to 10  $\mu\text{g/ml}$ . Wells were incubated for 96hrs at  $37^\circ\text{C}$  with 5%  $\text{CO}_2$  and the media was discarded. 100 $\mu\text{l}$  of MTT solution was added to each well and incubated for 4hrs. Blue colored formazon crystals formed by proliferating the cells were dissolved in DMSO under safety cabinet. Well plates were placed on a orbital shaker for 10 minutes at room temperature and absorbance of it was measured at 550nm by a microplate reader<sup>18,19</sup>.

**Tissue distribution studies :** Cell lines i.e., B16F10 were injected subcutaneously in balb/c mice respectively to form tumors. Animals were sacrificed and the tumors were propagated in adequate number of experimental animals. Animals bearing a tumor of 100mm<sup>3</sup> were starved overnight and dendrimer formulation containing 10mg/ml of drug-dendrimer formulation and drug were injected i.v. through tail vein. Animals were sacrificed at 10, 30, 60, 120 and 180 minutes after injection. Developed tumors and other organs like spleen, liver, kidney and lungs were isolated, washed with distilled water, weighed and homogenized in PBS to 1g/ml of tissue homogenate. Obtained homogenates were centrifuged at 1000rpm and the supernatants were analyzed in HPLC<sup>20</sup>.

**Pharmacokinetic studies :** All animal procedures were carried as per the protocol approved by CPCSEA on the experimenting animals. Pharmacokinetic studies were performed on B16F10 mice and they have been chosen as model animals for many pharmacokinetic and bioavailability studies. Animals under study were starved overnight. Control group was fed with saline and test group was given dendrimer drug formulation orally. Water was supplied throughout the course of experiment. Blood samples were withdrawn at fixed time intervals such as 0, 15, 30, 60, 120 and 180 minutes respectively. Collected samples were centrifuged at 20000rpm for 10 minutes. Supernatant plasma layer was collected into another tube for HPLC analysis and graphs were plotted against time<sup>20</sup>.

## Results and Discussion

**MTT assay :** Invitro acute cytotoxicity activity was performed against B16F10 and K562 cell lines by MTT assay. Concentration of the drug i.e., cyclophosphamide in the drug-dendrimer formulations ranged from 0.001 to 10  $\mu\text{g/ml}$ . At 0.001  $\mu\text{g/ml}$ , cellular proliferation inhibition was 10.47% whereas at 10  $\mu\text{g/ml}$  percentage inhibition was 62.49%. The cytotoxicity was thought to be a result of interaction between dendrimers and cell surfaces. In addition, there are several studies that prove higher generation dendrimers possess strong cytotoxicity when compared to other lower generations.

**Table 1. Tabular representation of MTT assay results against B16F10 cells**

Concentration ( $\mu\text{g/ml}$ )	DC	G5DDC	G5D
10	74.8	62.49	10.58
1	43.21	34.58	11.64
0.1	34.18	29.65	2.39
0.01	14.78	11.24	1.28
0.001	10.42	10.47	2.17
<b>IC50value</b>	<b>2</b>	<b>2</b>	<b>&gt;100</b>

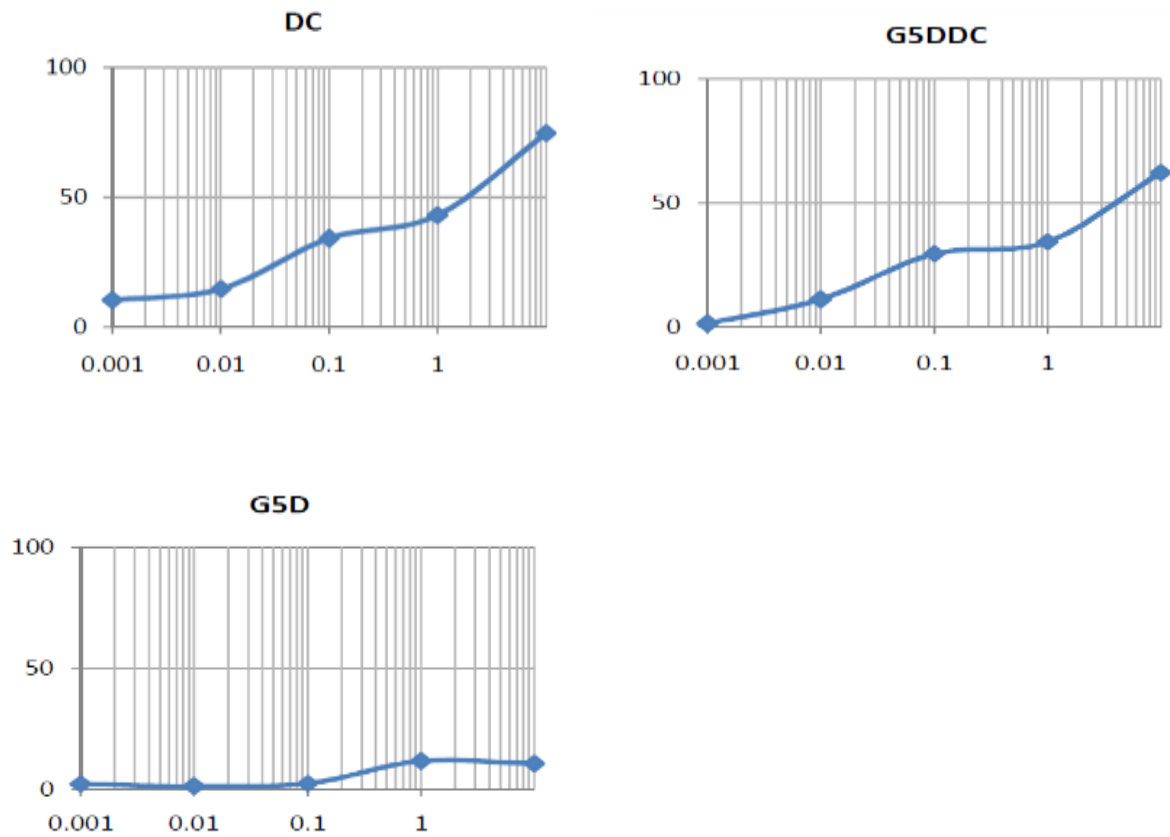
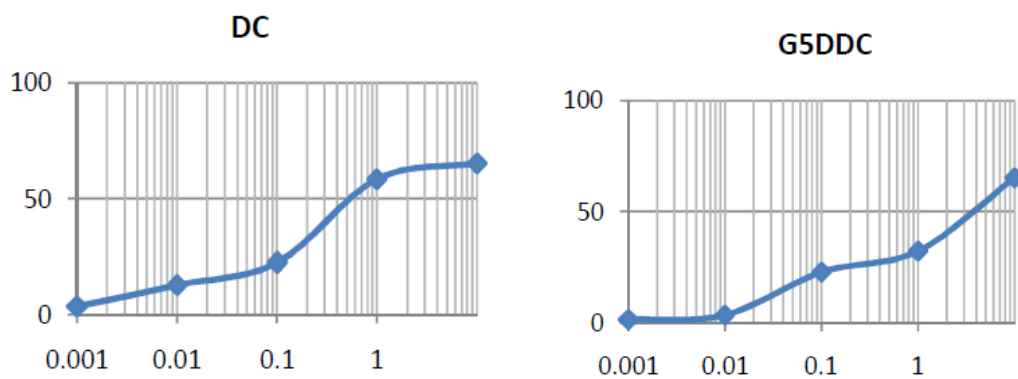
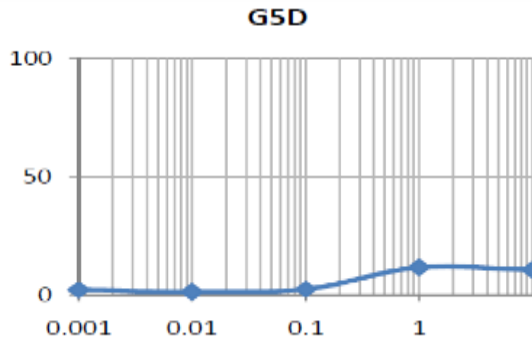


Fig no. 1 Graphical representation of MTT assay results for drug Cyclophosphamide for B16F10 cells

Table 2. Tabular representation of MTT assay results against K562 cells

Concentration (µg/ml)	DC	G5DDC	G5D
10	65.31	65.38	8.85
1	58.47	32.38	7.44
0.1	22.48	22.95	5.21
0.01	12.84	3.54	6.47
0.001	3.54	1.47	3.87
IC50value	0.5	4	>100





**Fig no. 2**Graphical representation of MTT assay results for drug Cyclophosphamide for K562 cells

**Tissue distribution studies :**

Tissue distribution study was performed on B16F10 mice. After administering drug, mice were sacrificed to study drug distribution pattern to various body organs such as tumor, liver, spleen, kidney,lungs. Results demonstrated that tumor uptake of cyclophosphamide-dendrimer formulation increased when compared to other organs. The reason behind the increased uptake of drug-dendrimer formulations by tumor is the enhanced permeation and retention effect. Results of the study are presented below.

**Table 3. Tabular representation of tissue distribution study data in tumor (µg/ml)**

Distribution time (min)	10	30	60	120	180
DC	4.86	10.06	13.5	12.5	12.52
G5DDC	5.33	9.95	18.02	16.7	17.2
G5D	0	0	0	0	0

**Table 4. Tabular representation of tissue distribution study data in lungs (µg/ml)**

Distribution time (min)	10	30	60	120	180
DC	5.27	7.76	12.36	13.58	11.75
G5DDC	5.33	8.82	15.3	15.10	15.0
G5D	0	0	0	0	0

**Table 5. Tabular representation of tissue distribution study data in liver (µg/ml)**

Distribution time (min)	10	30	60	120	180
DC	5.65	9.61	13.22	14.30	13.21
G5DDC	6.82	7.75	16.19	16.81	16.22
G5D	0	0	0	0	0

**Table 6. Tabular representation of tissue distribution study data in kidney (µg/ml)**

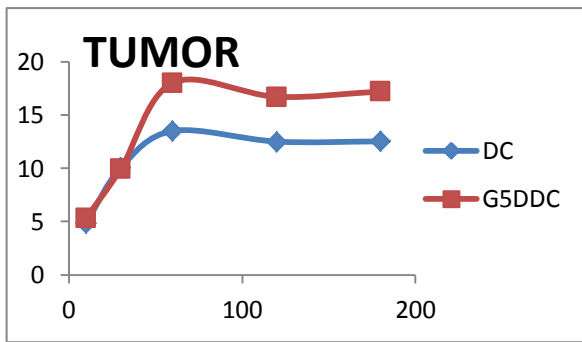
Distribution time (min)	10	30	60	120	180
DC	5.59	10.69	11.41	11.87	13.57
G5DDC	5.97	9.61	11.77	16.41	16.75
G5D	0	0	0	0	0

**Table 7.** Tabular representation of tissue distribution study data in spleen (µg/ml)

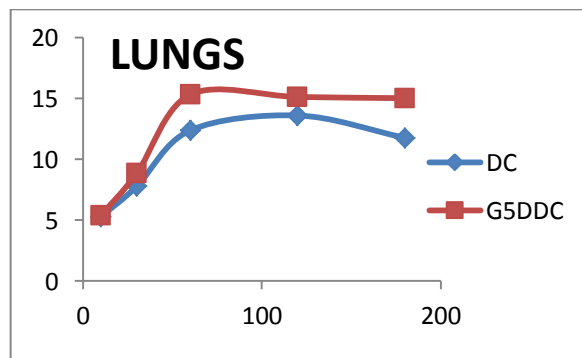
Distribution time (min)	10	30	60	120	180
DC	5.88	9.49	13.22	14.2	12.5
G5DDC	5.99	9.75	15.37	17.2	15.05
G5D	0	0	0	0	0

**Table 8.** Tabular representation of tissue distribution study data for plasma

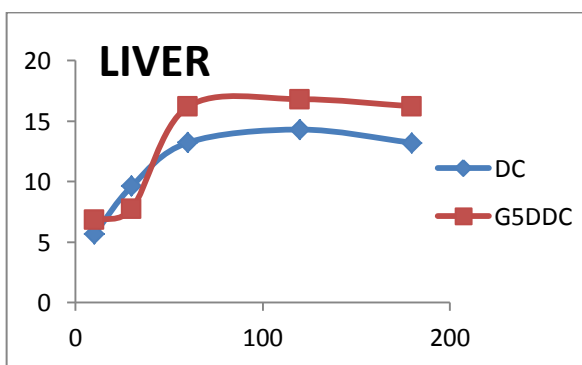
Distribution time (min)	10	30	60	120	180
DC	20.46	15.54	9.89	6.36	4.32
G5DDC	16.01	11.37	10.12	8.93	5.91
G5D	0	0	0	0	0



**Fig. 3** Graphical representation of tissue distribution study results in Tumor



**Fig.4** Graphical representation of tissue distribution study results in Lungs



**Fig.5** Graphical representation of tissue distribution study results in Liver

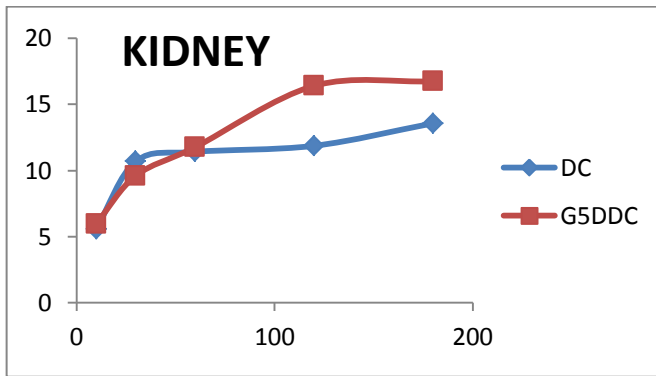


Fig. 6 Graphical representation of tissue distribution study results in kidney

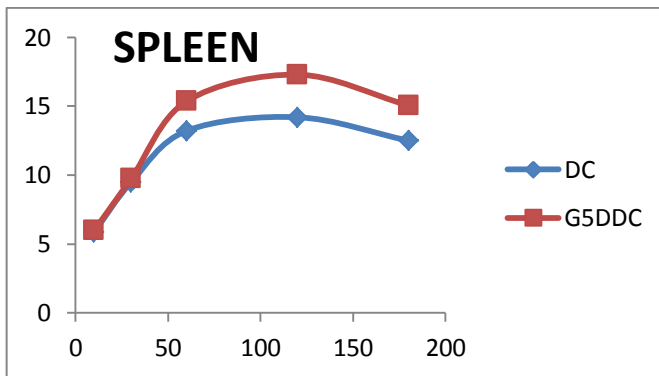


Fig. 7 Graphical representation of tissue distribution study results in Spleen

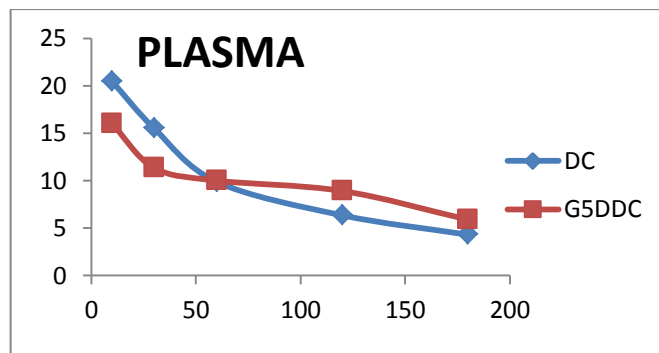


Fig. 8 Graphical representation of tissue distribution study results in plasma

**Pharmacokinetic studies :**

In-vivo pharmacokinetic studies were performed to quantify the amount of drug from the formulations after the iv administration of drug-dendrimer complex. Fig. 9 shows the mean plasma concentration – time curve of cyclophosphamide after its iv administration. Cmax of drug-dendrimer complex is 16.01 µg/ml and its AUC at the end of 30 minutes is 11.37 µg/ml.

**Table 9. Pharmacokinetic study parameters**

Parameters	Cmax (µg/ml)	Tmax (min)	T1/2
DC	20.46	30	110
G5DDC	16.01	30	110

Table 10. Plasma AUC

Distribution time (min)	10	30	60	120	180
DC	20.46	15.54	9.89	6.36	4.32
G5DDC	16.01	11.37	10.12	8.93	5.91
G5D	0	0	0	0	0

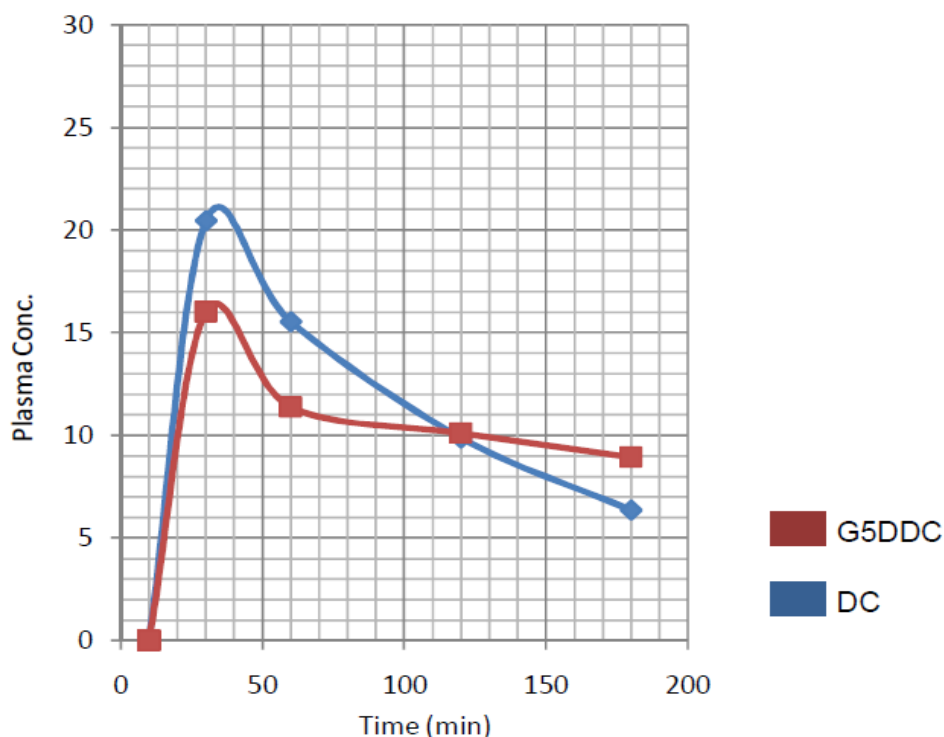


Fig. 9 Graphical representation representing plasma drug concentration

Highly branched citric acid dendrimer was found an effective carrier for encapsulating the hydrophobic moiety, cyclophosphamide. Distribution of drug through drug-dendrimer complex in the mouse body was time-dependent manner against B16F10 cells. When compared to other organs, cyclophosphamide uptake increased significantly in tumor cells and higher generation dendrimer exhibited strong cytotoxic activity when compared to other lower generations.

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### Conflict of Interest

All authors (B. NarasimhaRao, K. Ravindra Reddy, K. B. Chandra Sekhar) declare that they have no conflict of interest.



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