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CYTOTOXIC RESPONSE OF HCT116 CELL LINE TO MULTIFUNCTIONALIZED MWCNTS IRINOTECAN COMPLEX IN ASSOCIATED WITH MONOCLONAL ANTIBODIES

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ABSTRACT
Functionalized multi walled carbon nanotube drug delivery system for anticancer drug Irinotecan was designed, Carbon nanotubes functionalized prior to drug and antibodies binding, which makes under biological conditions, multiple functionalization done by binding Bevacizumab antibodies to functionalized multi walled carbon nanotube Irinotecan complex, which promotes through active targeting. Present work is an attempt to investigate the cytotoxic studies of different functionalized MWCNTs by MTT assay. HCT116 were seeded in a 96 well plate, the seeded HCT 116 cells were treated with all the prepared functionalized MWCNTs formulations, MTT assay procedure followed to determine the cell viability of HCT 116 cells. Cells were treated with 10 µL of the formulations, 20 µL of MTT (1 mg/mL) was added to each well and incubated for 3 hours, and then MTT is removed completely from each well, the absorbance was measured at 570 nm to detect the cell viability and cell death. MMS demonstrated an 82.33 % cell death. MWCNTs Covalent functionalized Irinotecan conjugate, MWCNTs Non Covalent functionalized Irinotecan conjugate, MWCNTs Covalent and Non Covalent functionalized Irinotecan conjugate, MWCNTs and both Covalent and Non Covalent functionalized Irinotecan Bevacizumab Antibodies conjugate were cytotoxic with percentage of cell death 52.21 %, 55.15 %, 59.23 % and 73.98 % respectively at 62.5 µg/mL concentration. All the prepared formulations can be used as adjunct therapy for major cancer treatment such as Surgery, radiotherapy and chemotherapy.

KEYWORDS: MWCNTs, MTT Assay, HCT116 Cells, Bevacizumab antibodies, Cytotoxicity, Irinotecan.

INTRODUCTION
The uncontrolled proliferation of cells that destroys normal tissues and organs is known as cancer. Cancer is a catch-all word for the leading cause of death in modern society. According to the World Health Organization, millions of people died worldwide in 2020.

Carbon is the 15th most prevalent element in the earth's crust. Because of its tetra valency, sp3 can hybridize in carbon and produce a wide range of crystalline forms. Different allotropic forms of carbon include graphite, diamond, Buckminster fullerenes, carbon nanotubes (CNTs), graphene, and around 500 speculative allotropes.[1,2] CNTs, in particular, have sparked a lot of interest in nano-carbon-based composites for drug delivery.[3-6]

Carbon nanotubes are made from graphene planes that have been rolled. CNTs have been widely used in biomedicine due to their unique chemical and physical properties, as well as their nanostructures. Iijima discovered CNTs in 1991 while doing experimental and conceptual research with High Resolution Electron Microscopy (HREM). CNTs have shown to be a versatile tool with numerous applications. They are technologically and scientifically significant in a variety of ways, ranging from delivery systems to competitiveness in targeting. The angle and diameter of CNTs have a big impact on their incredible functionality. CNTs are allotropes of carbon with a nanostructure and a length-to-diameter ratio greater than 1,000,000. For creative uses of CNTs, chirality, diameter, number of layers and purity are important factors to consider. Unique CNTs could be exploited mechanistically to

deliver chemotherapy drugs to the tumour site by manipulating this features.[7-8]

Single-walled (SWCNTs) and multi-walled (MWCNTs) carbon nanotubes structures are endowed with a high aspect ratio are ultra-lightweight, have great strength, have good thermal conductivity, and have electrical properties that range from metallic to semiconducting. Single walled CNTs have single graphite sheets with diameters ranging from 0.4 to 3 nm, and multi walled CNTs have diameters ranging from 2 to 100 nm and lengths ranging from 1 to 50 μm.[9,10]

CNTs can be made using plasma-based synthesis, arc discharge evaporation, laser ablation, thermal synthesis, chemical vapour deposition, and plasma-enhanced chemical vapour deposition. The fields which are benefited from CNTs are drug delivery, blood cancer, breast cancer, brain cancer, liver cancer, cervical cancer, gene therapy, immunological therapy, biomedical imaging, biosensors, and tissue engineering.[11-14] CNTs were manufactured using a variety of procedures designed for fabricating CNT structures that require gas phase processes. The carbon carbon arc-discharge technique, the laser ablation, chemical vapor deposition (CVD) technique are common methods.[15]

In the field of nanotechnology, functionalization of carbon nanotubes (f-CNTs) is emerging as a novel tool. CNTs are materials that are nearly insoluble in any solvent or are rarely dispersed in any solvent. To incorporate nanotube technology into the biological environment, the tubes' solubility, particularly in aqueous solutions, must be increased. Individual tubes as influenced by the attractive van der Waals contact bundling/aggregation activity is reduced. It may enable efficient intracellular absorption and better efficacy, allowing CNTs to be internalized more effectively. It could make it easier to connect different functional groups to the surface of CNTs for biomedical purposes. Chemical modification of Multi-Walled Carbon Nanotubes (MWCNTs) is recognized as an effective method for functionalization, promoting dispersion and surface activation at the same time to overcome self-aggregation.[16-18]

Covalent or noncovalent techniques could be used to functionalize CNTs.[19-20] For CNT modification at the sidewall and tip area, several organic chemicals have been utilized in covalent functionalization[49-54] by two main strategies “end and defect” and “sidewall”. Surfactants, nucleic acids, peptides, polymers, and oligomers are used to noncovalently functionalize CNTs. The electrical structure of the nanotube aromatic surface is preserved as a result of this operation. CNTs are split and oxidised to produce a certain quantity of CNTs, which are then derivatized with various compounds. CNT sidewalls can also be directly functionalized through addition processes. The addition of moieties to the tube's exterior surface causes repulsion between the individual tubes, allowing them to disperse freely into the solvent.[21-23]

The current research project focuses on the fine-tuning of MWCNT pre functionalization via covalent functionalization by acid reflux, noncovalent functionalization by PEG, and a combination of these two. Anticancer drug irinotecan is attached to functionalized MWCNTs for drug delivery applications. Bevacizumab antibodies were used to bind the covalent and noncovalent functionalized MWCNTs Irinotecan complexes independently. As part of the biological evaluation, all of the formulations were tested for cytotoxicity (screening assays). Chemotherapeutic drugs must be evaluated using cell-based assays that measure anticancer drug effects. To determine the specificity of Irinotecan attached to functionalized MWCNTs in causing cancer cell apoptosis, it is necessary to assess their anticancer activities. In present research work the developed Multiple functionalized MWCNTs Irinotecan complexes were investigated for cytotoxicity assessments.

I) MATERIALS AND METHODS

Multiwalled carbon nanotube (outer diameter 10- 30 nm, number of walls 5-15, length 1- 10μm) was purchased from Nano Wings Private limited, Telangana. Irinotecan was obtained as gift sample from Nantong Jinghua Pharmaceutical co., ltd, Jiangsu, China. Methanol, Hydrochloric acid, Sulphuric acid, Nitric acid, Ammonium hydroxide (25 %), Hydrogen peroxide (30%), PEG etc… are Laboratory grade, they were kind gift from Samarth life science private limited, Bevacizumab antibodies was a kind gift from Biocon Biopharmaceuticals, Bommasandra, Bangalore. HCT116 cells cultured and MTT Assay were carried out at Anthem Biosciences, Bangalore.

ii) Procedure for Irinotecan formulations[24-29]

I. Covalent functionalization process

The following three methods are adapted to obtain covalent functionalized MWCNTs from pristine MWCNTs.

a) Initial acidic treatment followed by treatment with hydrochloric acid: Take 250 mg of Pristine MWCNTs in to conical flask add 100 ml of 65% HNO_3 and 98% H_2SO_4 acids were used for the initial acidic treatment which leads to produce oxidized MWCNTs. The obtained oxidized MWCNTs were treated The initial acidic treatment done by using HNO_3 and H_2SO_4, which produces oxidized MWCNTs and then same oxidized MWCNTs are treated with HCl and produces carboxylated MWCNTs. Take 250 mg of MWCNTs in flask add 100 ml mixture of 98 % H_2SO_4 and 65 % HNO_3 (V:V = 3:1) and the above mixture subjected to 12 h agitation at room temperature, oxidised MWCNTs were obtained. Oxidized MWCNTs were washed with fresh water and add HCl, keep the mixure 24 h for reflux, then filtered to obtain carboxylated MWCNTs and wash

the mixture with fresh water until to neutral pH. The filtered products are dried overnight at 40 ^{o}C in vacuum.

b) Treatment with concentration Hydrochloric acid: In order to purify the MWCNTs treatment with concentration Hydrochloric acid method is followed. In 100 ml beaker take 250 mg of MWCNTs mix with 100 ml of HCl, the resultant mixture stirred with the help of magnetic stirrer for 2 h, treat with sufficient fresh water until to neutral pH and then filter and dry overnight in vacuum at 40 ^{o}C.

c) Initial basic treatment followed by treatment with hydrochloric acid: To fabricate covalent functionalized MWCNTs Initial basic treatment followed by treatment with hydrochloric acid method is followed. The initial basic treatment for MWCNTs were done by taking 250 mg of MWCNTs in a round bottom flask with condenser, add 12.5 ml of ammonium hydroxide (25 %) and hydrogen peroxide (30%) (V: V=1:1) to obtain oxidized MWCNTs. HCl is refluxed for 5h with oxidized MWCNTs and heated to 80 ^{o}C to produce carboxylated MWCNTs, add fresh water until to neutral pH and filter, the obtained residue was washed with fresh water and dry the residue over night in vacuum at 40 ^{o}C.

II. Non covalent functionalization

Take 1 g of PEG 4000 and 10 ml of distilled water in tarson tube, add 500 mg of pristine MWCNTs in to PEG solution, then subject to sonication for binding of PEG with the help of fast clean ultra sonic bath sonicator for 15 mins. Removal of unbound PEG and agglomerated MWCNTs were accomplished by centrifugation at 7000 r/5m. Discard unbound PEG supernatant liquid, filter the ppt by using 0.2 micro filters (Mililpore) by applying vacuum and dry the PEG functionalized MWCNTs in room temperature.

III. Pristine MWCNTs were prefunctionalized by using above methods for binding Irinotecan and Bevacizumab antibodies. Functionalization process carried out by covalent, noncovalent and combination of covalent and noncovalent functionalization, anticancer drug Irinotecan binded to above functionalized MWCNTs, Bevacizumab antibodies were loaded on covalent and non covalent functionalized MWCNTs Irinotecan formulation as given in table 1. Cell cytotoxicity properties of all formulations given Table 1 were evaluated using MTT proliferation assay using HCT116 cancer cells in order to determine the anticancer effects of the nanoconjugates *in vitro*.

Fig. 1: Irinotecan Formulations.

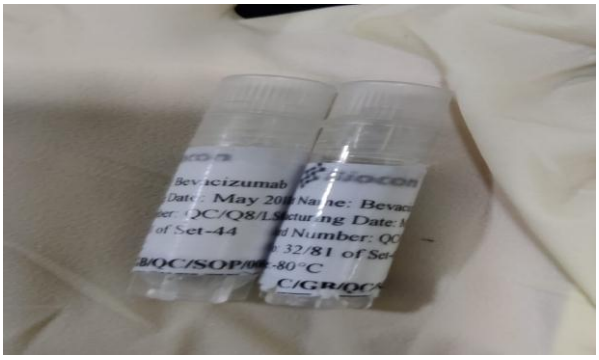


Fig. 2: Bevacizumab Antibodies

Table 1: Irinotecan formulations.

Formulations		Functionalization method	Drug	Antibodies
1	**Control**	_	Irinotecan	
2	**Control**			Bevacizumab antibodies
3	MWCNTs	Covalent Functionalization	Irinotecan	
4		Non- Covalent Functionalization	Irinotecan	
5		Covalent +Non- covalent Functionalization	Irinotecan	
6		Covalent +Non- covalent Functionalization	Irinotecan	Bevacizumab antibodies

c) Preparation of Functionalized MWCNTs formulations for cell viability[29-31]

In our research work, all MWCNTs conjugate formulations were prepared by functionalization followed by binding drug and Bevacizumab antibodies depicts in table 1. All prepared MWCNTs conjugates were tested for cytotoxicity by treating on HCT 116 cells. Stock concentration of each MWCNTs conjugate formulations were prepared according to given in table 2, stock concentration prepared in Milli Q water, sonicate for 15 minutes and dilute subsequently dilute the formulations to the required doses via serial dilutions. Before the treatment to HCT 116 cells the formulations were subjected to agitation for 2 minutes. The different MWCNTs conjugate formulations with different doses were treated with HCT116 cells, incubated for 72 hours and assess cytotoxicity.

Cytotoxicity assay

Cytotoxicity assay was carried out to analyze the effect of the all MWCNTs Irinotecan conjugate formulations on HCT116 cells. The set of cells were treated with the Irinotecan formulations as given in table 2 and incubated for 72 hours at 37 ˚C with 5% CO2. To measure cytotoxicity, MTT reagent was added, by considering the vehicle control growth as 100 %, the death percentage was determined.

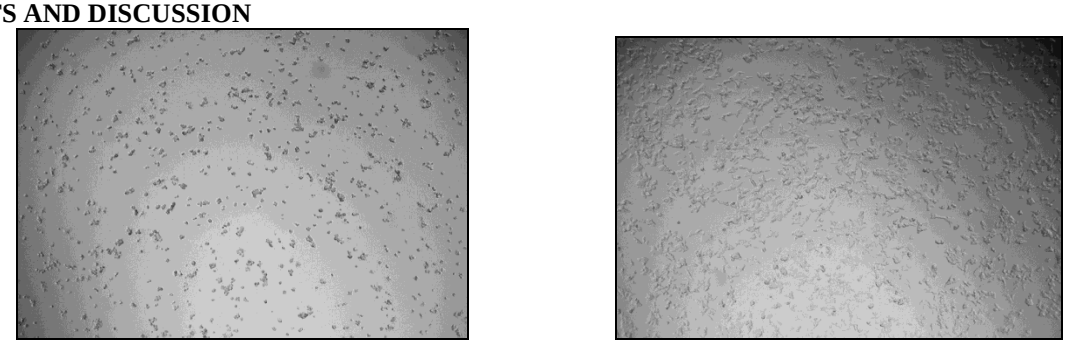
ii) Cell Culture

In T -25 and T-75 cell flasks, HCT 116 cells will be revived, cultured and maintained to a confluency 80 to 90 %. The cells were seeded in a 96-well plate and incubated till the cells were adhered at a density of 10,000 cells per well with 5% $C0_2$ at 37 ºC for overnight. After 72 hours incubation, the plates will be treated with resazurin (Alamar blue) or equivalent reagent (MTT) for 3 hours to determine the viability of cell. MMS will be used as positive control. The cell viability will be determined by considering the 100% viability of the cell growth in the vehicle control. The cell viability will be evaluated in triplicate 0-8 dilutions/ drug/cell line at one-time point, tetrazolium salts or resazurin or in equivalent used. Tumor cell growth is most apparent and rapid growth level is maintained. HCT 116 Cells were treated with 10 µL of the MWCNTs conjugate formulations/ MMS (Semi log dilutions). Cells and culture medium treated with Milli Q water to maintain as vehicle control for the compound and MMS respectively. The plate were incubated with 5%CO2 for 72 hours at 37 ºC. Medium containing MTT reagent was completely detected and 100 µL of 100% DMSO was added from each well. The cell viability and cell death was measured by absorbance at 570 nm.

Table 2: Details of Stock Concentration of all Irinotecan formulations.

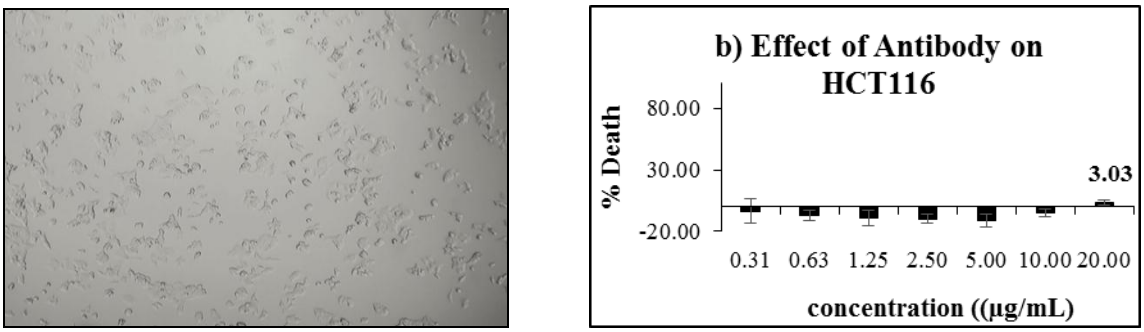
Sl no	Compound	Stock concentration	Solvent used to make the stock and working concentration
1	Irinotecan Control	20 mg/mL	Milli Q water
2	Covalent functionalized Irinotecan	20 mg/mL	Milli Q water
3	Non Covalent functionalized Irinotecan	20 mg/mL	Milli Q water
4	Covalent and Non covalent functionalized Irinotecan	20 mg/mL	Milli Q water
5	Covalent and Non covalent functionalized Irinotecan Antibody conjuagte	20 mg + 2 µg/mL	Milli Q water
6	MMS (Positive control)	11.68 M	

RESULTS AND DISCUSSION



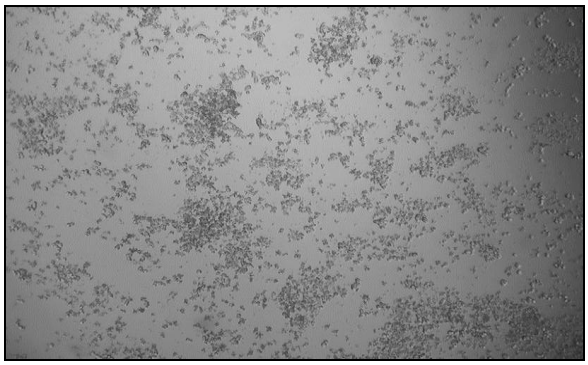
a)500 µg/mL Precipitation observed **b) Vehicle Control (Cell culture medium)**

Fig 3: Cytotoxicity of MMS on HCT116.

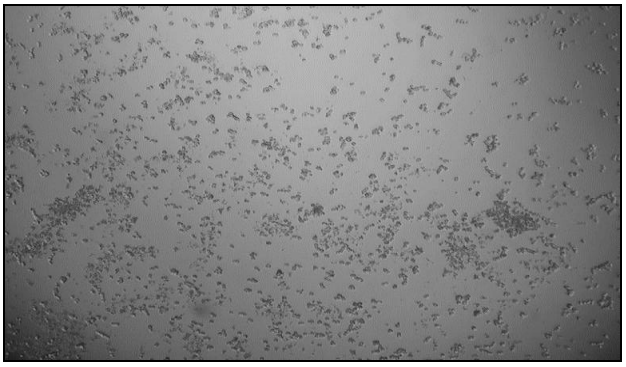


a)images of antibodies with the cells no precipitation observed **b) Effect of Antibody on HCT116 cells**

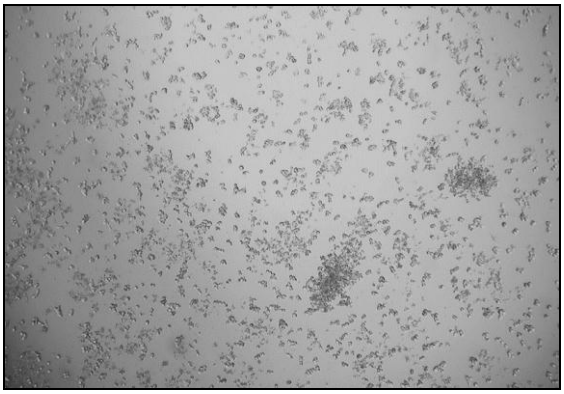
Fig 4: Cytotoxic images and graph of Bevacizumab Antibodies on HCT116 Cells.



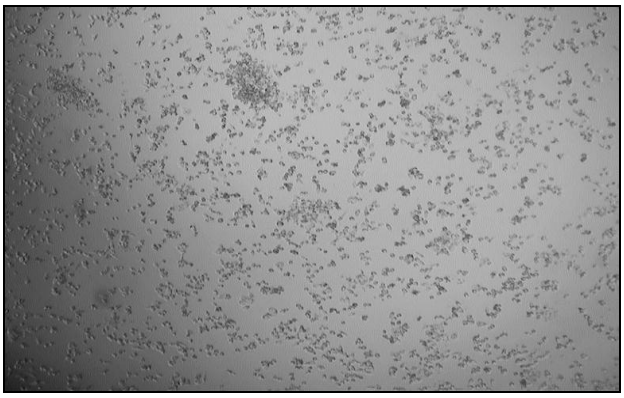
a)2000 µg/mL Precipitation observed



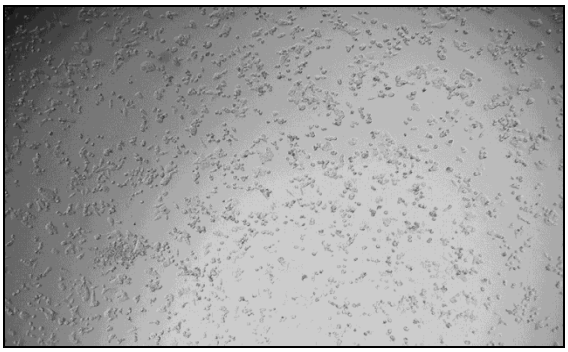
b) 1000 µg/mL Precipitation observed



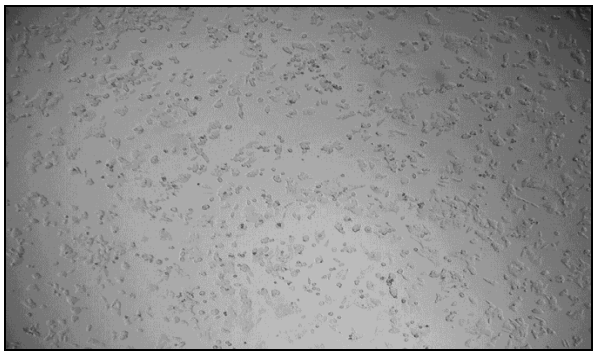
c) 500 µg/mL Precipitation observed



d) 250 µg/mL Precipitation observed

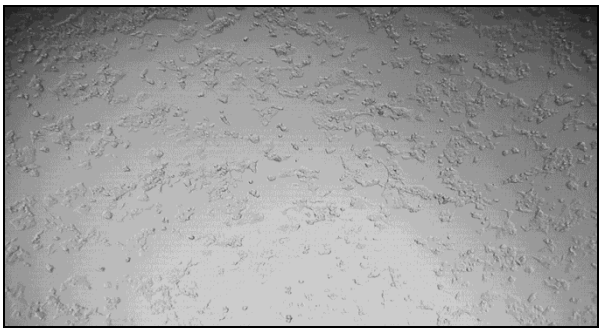


e) 125 µg/mL Precipitation observed



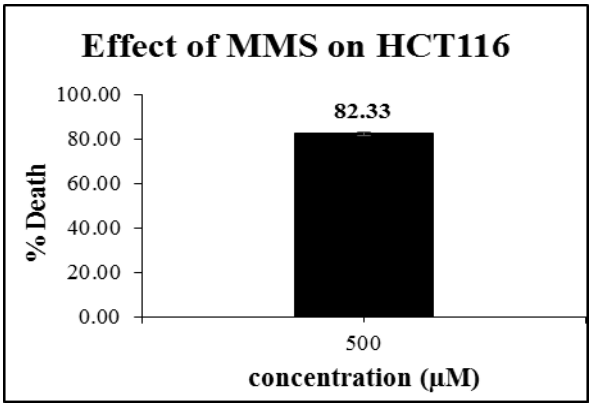
f) 62.5 µg/mL Precipitation observed

g)31.25 µg/mL Precipitation observed

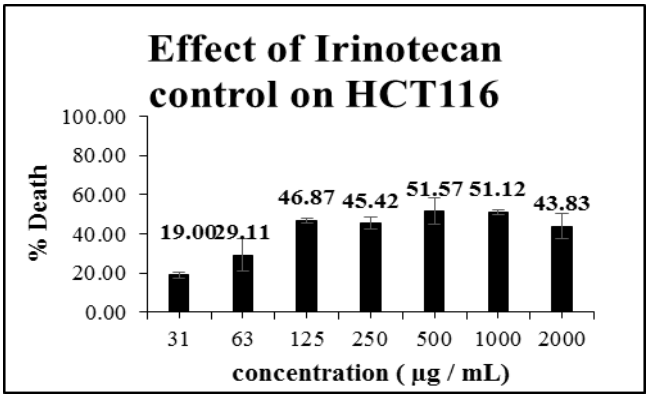


h) Vehicle Control (Distilled Water)

Fig.5 (a- h): Cytotoxicity images of Irinotecan Control on HCT116 at different concentrations and vehicle control.

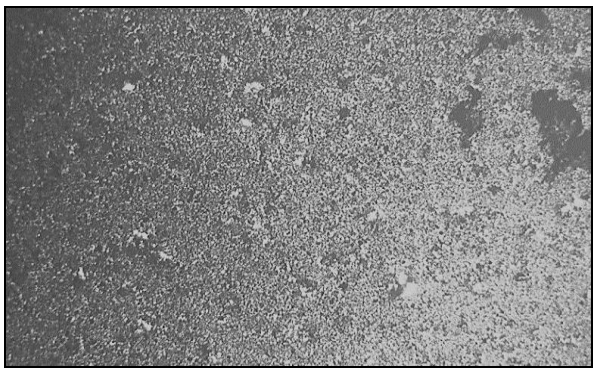


The positive control, MMS, exhibited 82.33 % death at 500 µM

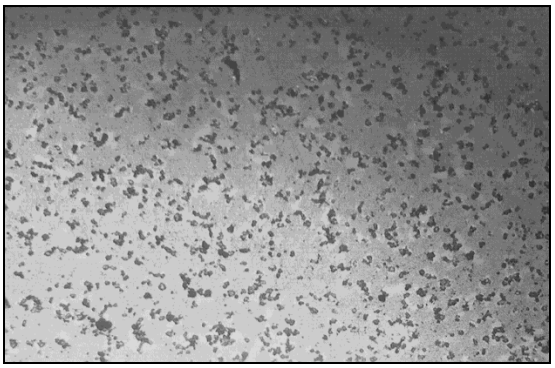


The Irinotecan control exhibited 50% cytotoxicity from 500 µg/mL concentration

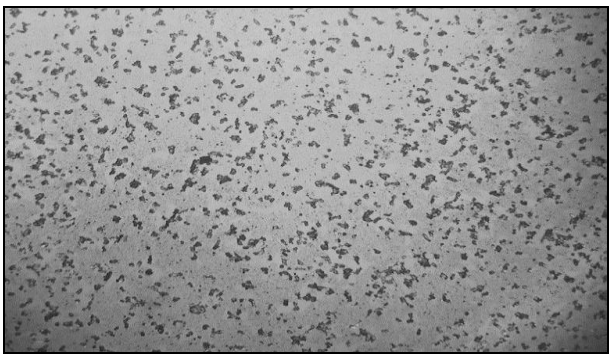
Fig. 6: Cytotoxicity graphs of MMS and Irinotecan Control on HCT116 cells at different concentrations.



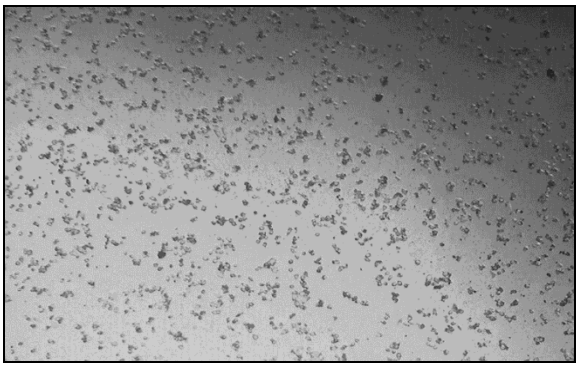
a)2000 µg/mL Precipitation observed



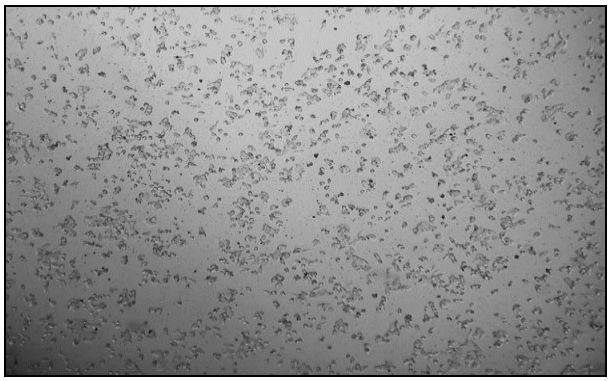
b) 1000 µg/mL Precipitation observed



c)500 µg/mL Precipitation observed



d) 250 µg/mL Precipitation observed

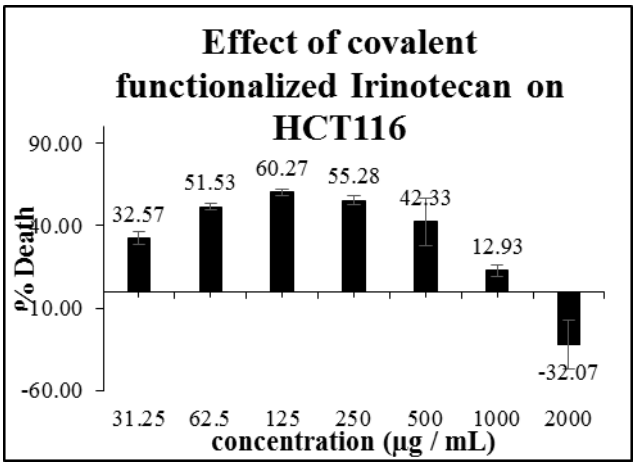


e)31.25 µg/mL Precipitation observed

f) Vehicle Control (Distilled Water)

Fig. 7 (a-h): Cytotoxicity of covalent functionalized Irinotecan conjugate on HCT116 cells at different concentrations.

Without normalization



With normalization

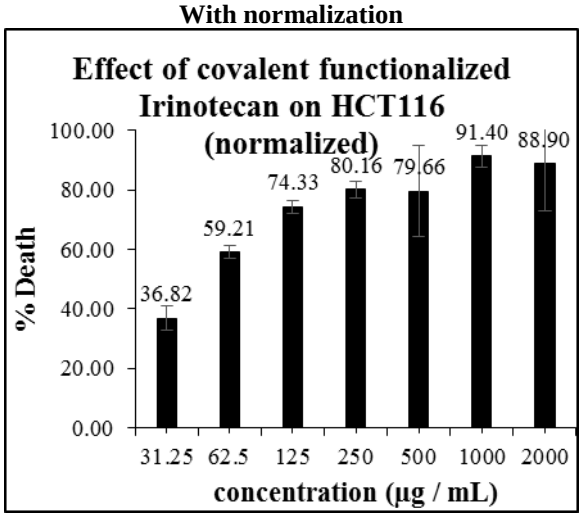
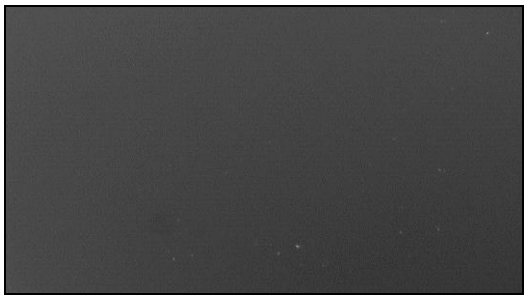
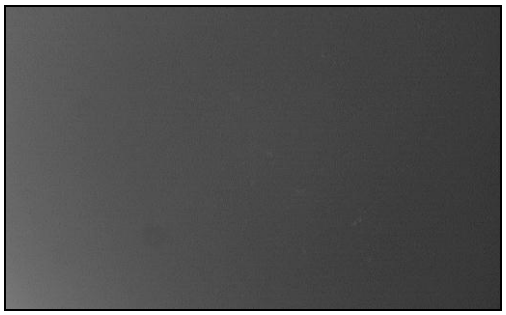


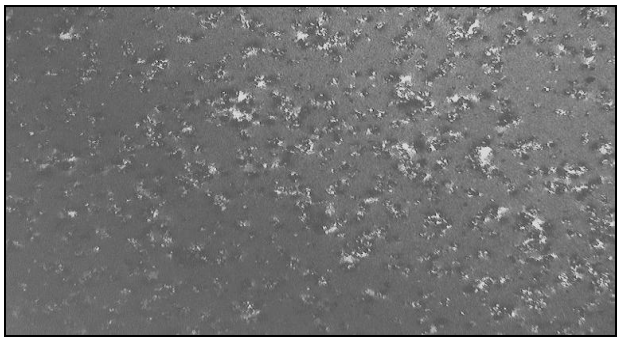
Fig.8: Cytotoxicity graphs of covalent functionalized Irinotecan conjugate on HCT116 cells at different concentration.



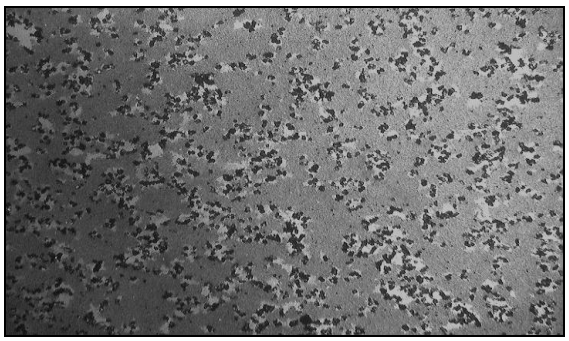
a) 2000 µg/mL Precipitation observed



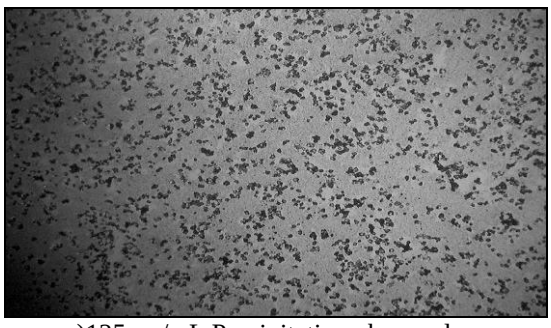
b) 1000 µg/mL Precipitation observed



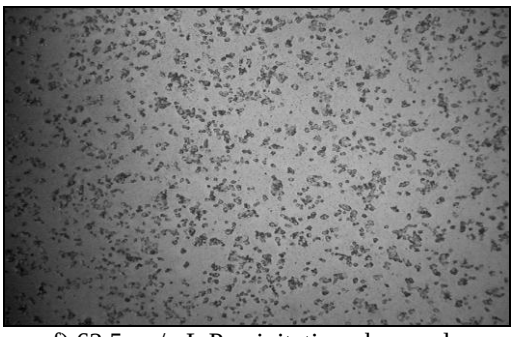
c)500 µg/mL Precipitation observed



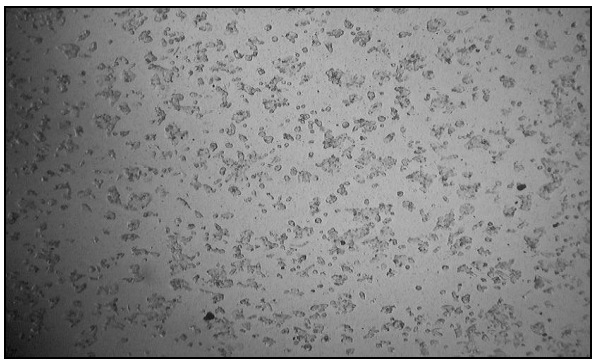
d) 250 µg/mL Precipitation observed



e)125 µg/mL Precipitation observed



f) 62.5 µg/mL Precipitation observed

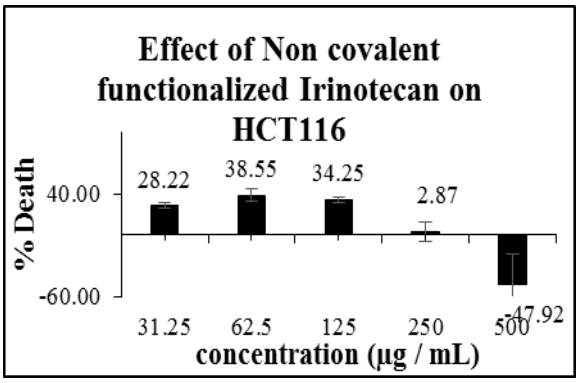


g) 31.25 µg/mL Precipitation observed

h) Vehicle Control (Distilled Water)

Fig. 9 (a-h): Cytotoxicity of Non covalent functionalized Irinotecan conjugate on HCT116 cells at different concentrations.

Without normalization



With normalization

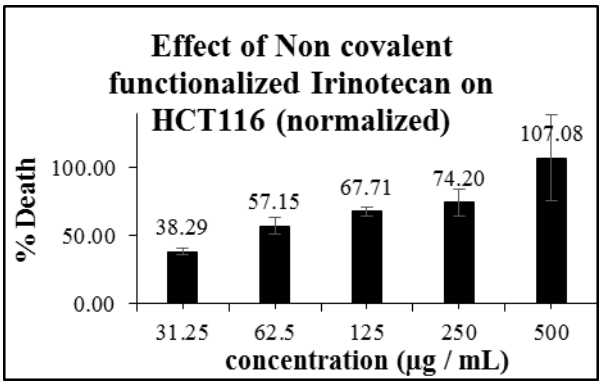
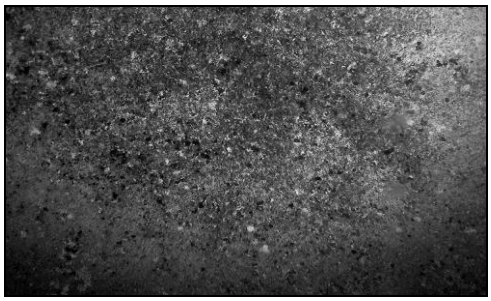
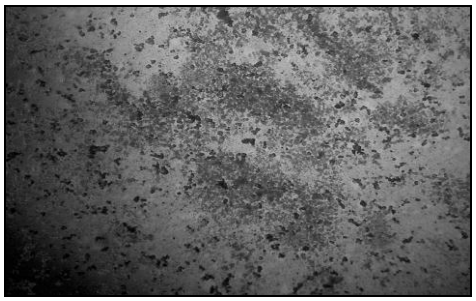


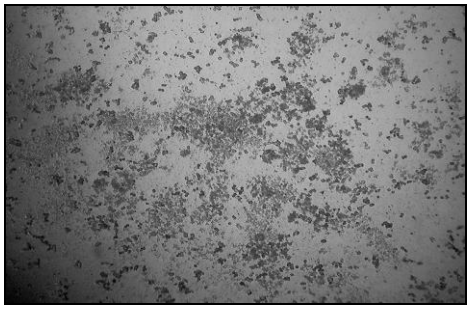
Fig. 10: Cytotoxicity graphs of Covalent and Non covalent functionalized Irinotecan conjugate on HCT116 at different concentrations.



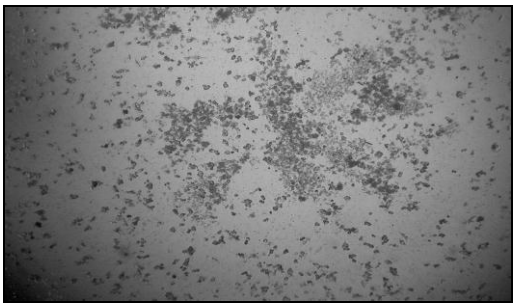
a) 2000 µg/mL Precipitation observed



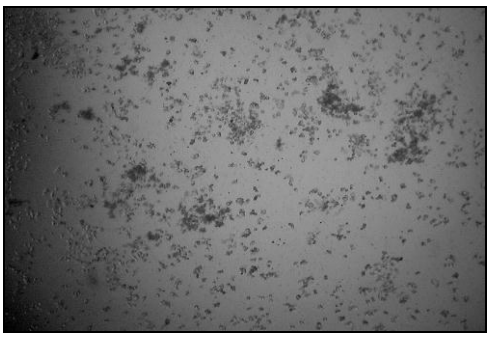
b) 1000 µg/mL Precipitation observed



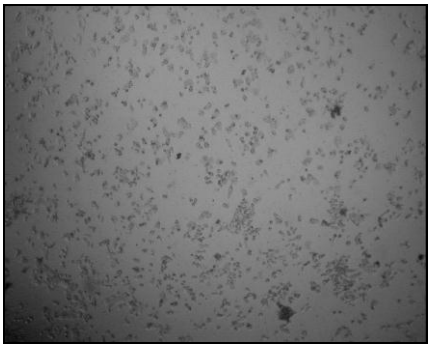
c) 500 µg/mL Precipitation observed



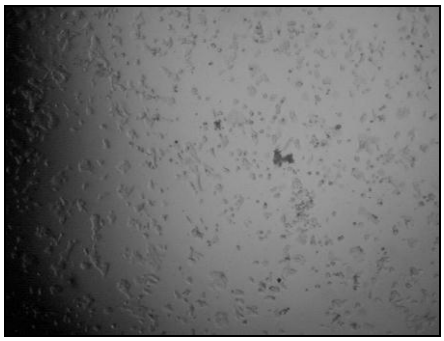
d) 250 µg/mL Precipitation observed



e)125 µg/mL Precipitation observed



f) 62.5 µg/mL Precipitation observed

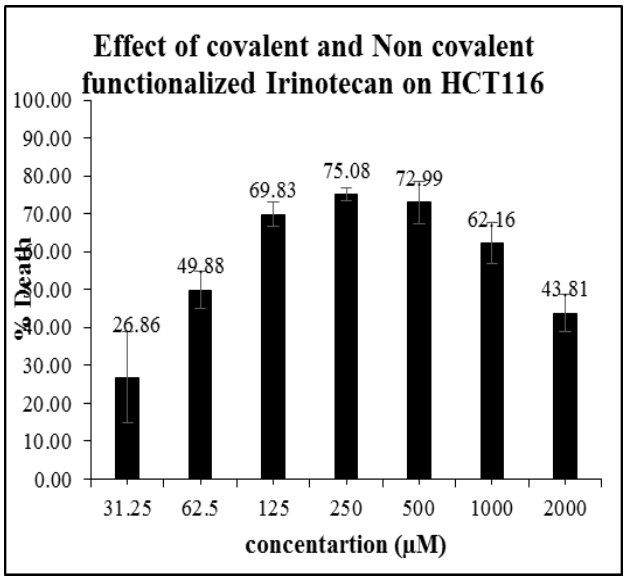


g)31.25 µg/mL Precipitation observed

h) Vehicle Control (Distilled Water)

Fig. 11 (a to h): Cytotoxicity of covalent and Non covalent functionalized Irinotecan conjugate on HCT116 cells at different concentrations.

Without normalization



With normalization

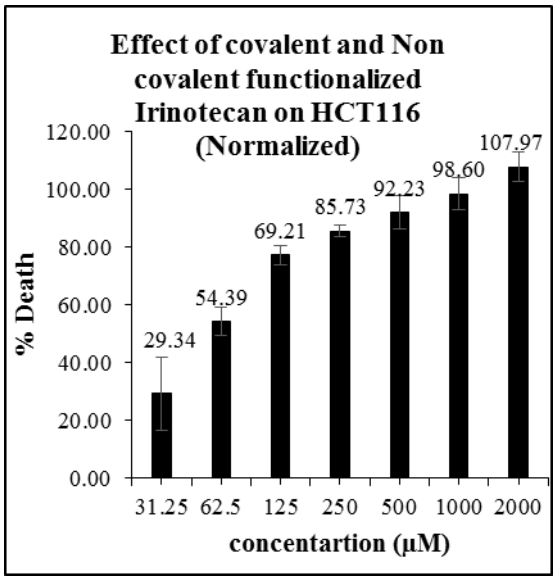
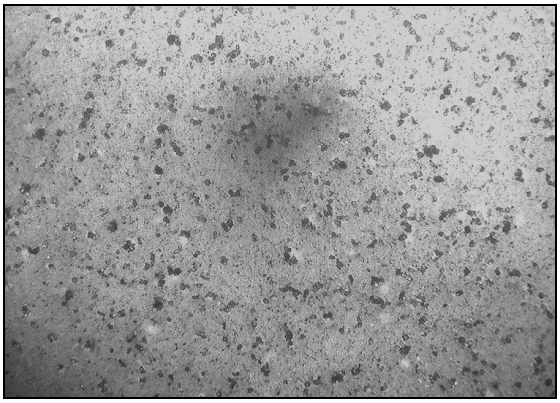
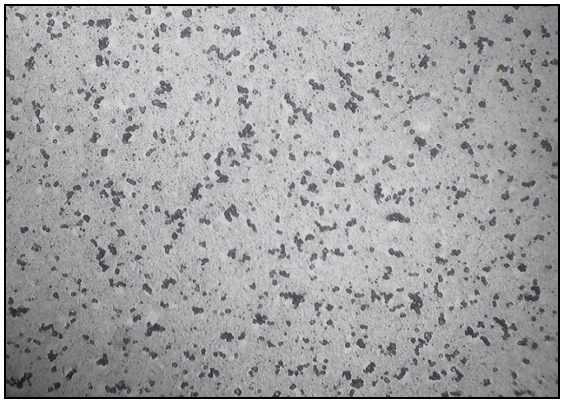


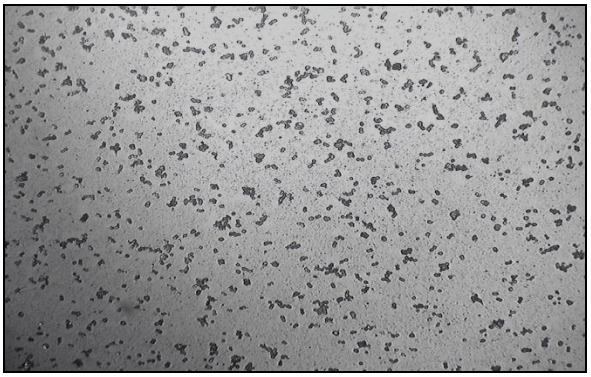
Fig.12: Cytotoxicity graphs of covalent and Non covalent functionalized Irinotecan conjugate on HCT116 cells at different concentrations.



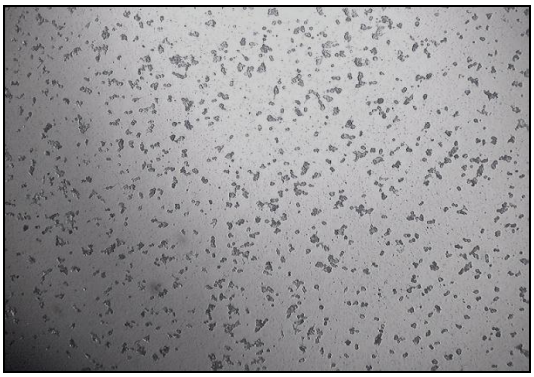
a)2000 + 20 µg/mL Precipitation observed



b)1000+10 µg/mL Precipitation observed



c)500+5 µg/mL Precipitation observed



d)250+2.5 µg/mL Precipitation observed

e)125+ 1.25 µg/mL Precipitation observed

f) 62.5+0.5 µg/mL Precipitation observed

g)31.25 µg/mL Precipitation observed

h) Vehicle Control (Distilled Water)

Fig. 13 (a-h): Bevacizumab Antibodies conjugate on HCT116 cells at different concentrations.

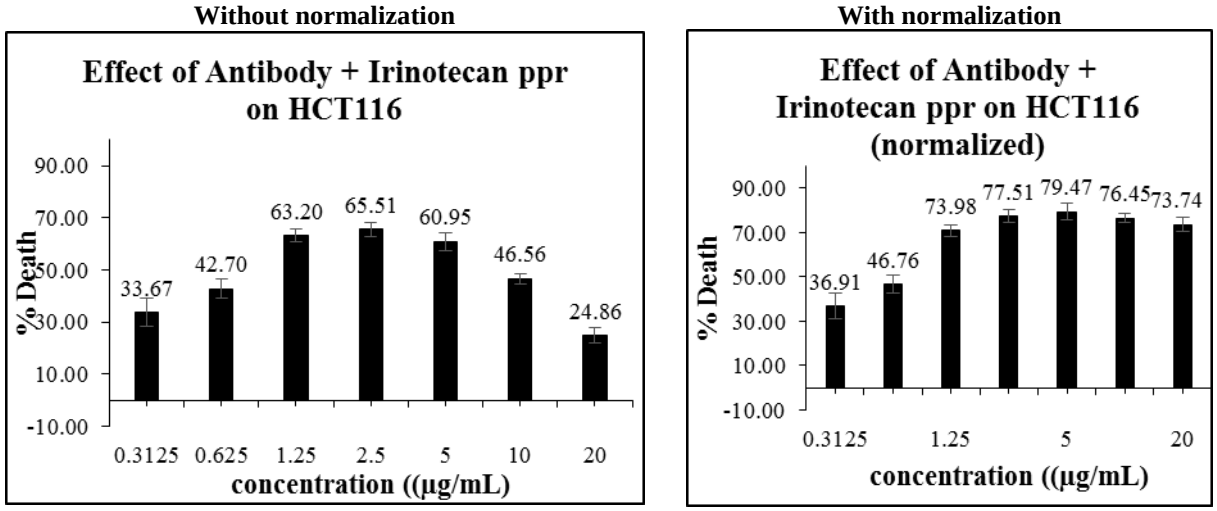


Fig. 14: Cytotoxicity graphs of covalent and Non covalent functionalized Irinotecan Bevacizumab Antibodies conjugate on HCT116 cells at different concentrations.

All the conjugates were tested for cytotoxicity on HCT 116 cells. MMS was taken as positive control and at the concentration of 500 µM it demonstrates an 82.33% death. The Irinotecan control exhibited 50% cytotoxicity with dose dependent cytotoxicity at 8 different dilutions and shows the cell viability or precipitations. Precipitation was observed for all the test compounds except Bevacizumab Antibodies control. The values were normalized with the absorbance values of compounds serially diluted (compound control). Post normalization with MWCNTs Covalent functionalized Irinotecan conjugate was cytotoxic and negate precipitation observed with 52.21 % cell death at 62.5 µg/mL concentration. MWCNTs Non Covalent functionalized Irinotecan conjugate was cytotoxic and exhibited 55.15% cell death at 62.5 µg/mL concentration, post normalization with compound only control to negate precipitation observed with the compound. Cytotoxic precipitation images of 8 different concentrations of MWCNTs Covalent and Non Covalent functionalized Irinotecan conjugate was cytotoxic and exhibited 59.23 % cell death at 62.5 µg/mL concentration, MWCNTs Covalent and Non Covalent functionalized Irinotecan conjugate was cytotoxic and exhibited 59.23% cell death at 62.5 µg/mL concentration, post normalization with compound only control to negate precipitation observed with the compound. MWCNTs Covalent and Non Covalent functionalized Irinotecan Bevacizumab Antibodies conjugate was cytotoxic and exhibited 73.98 % cell death at 62.5 µg/mL and antibodies 1.25 µg/mL concentration, post normalization with compound only control to negate precipitation observed with the compound. It was observed that MWCNTs formulations demonstrated that the significant cell growth inhibitory to HCT116 cells in a concentration-dependent manner and growth inhibitory activity observed by precipitation. Cytotoxicity of Functionalized MWCNTs Irinotecan formulations were evaluated in HCT116 cells using MTT assays. It was shown that Functionalized MWCNTs Irinotecan Bevacizumab Antibodies conjugates had much greater cytotoxicity compared to Functionalized MWCNTs Irinotecan formulations without antibodies. This indicates that Functionalized MWCNTs Irinotecan Bevacizumab Antibodies conjugates is potentially useful for the delivery of therapeutic agents Irinotecan. The employment of nanomaterials can improve the pharmacokinetic properties of anticancer agents and provide effective and selective treatment for the soluble, reactive different functionalized MWCNTs formulations was used as the starting point to build multifunctional constructs with appended Antibodies to affect specific targeting HCT116 cells, to carry and deliver Irinotecan drug.

CONCLUSION

In our research work, we have accomplish a few important goals such as MWCNTs were functionalized by different method process, loading of Irinotecan drug to functionalized MWCNTs and binding Bevacizumab Antibodies to both covalent and noncovalent functionalized MWCNTs Irinotecan conjugate. This will enhance the pharmacological therapy of anticancer drug Irinotecan will only be released to tumour, but not in healthy tissues by using a simple preparation method. Cell cytotoxicity assay of all Functionalized MWCNTs Irinotecan formulations indicating a satisfying biocompatibility and antitumor activity. The ability to specifically target tumor cells in vitro Antibodies appended Functionalized MWCNT constructs was encouraging and suggested. Fuctionalized MWCNTs Irinotecan Bevacizumab Antibodies conjugate treatment is an effective inducer of a hypoxic environment, but the resulting cell kill and tumor shrinkage by precipitation is determined by the susceptibility of the cells. Bevacizumab Antibodies along with chemotherapeutic agents binds to MWCNTs, attacks the cells more directly or connect with the HCT116 cells to deliver the treatment directly to the cancerous cells while avoiding healthy cells. All Functionalized MWCNTs Irinotecan conjugates effects shown up to 72 hr that confirms drug released in a sustained manner.

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