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# FABRICATION OF GELATIN FUNCTIONALIZED CARBON NANOTUBES SYSTEM FOR THE SITE-SPECIFIC DELIVERY OF CURCUMIN TOWARDS BREAST CANCER CELL

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## Abstract

The objective of the current research was to formulate SWCNTs anchored curcumin utilizing gelatin via carbodiimide conjugation for efficient targeting against human breast cancer celllines (MDA-MB-231). The Cur-Gel-CNT conjugate was formulated and evaluate the conjugation pattern by FTIR analysis. The system was also be characterized by various parameters such as zeta potential, particle size, transmission electron microscope (TEM), X-ray diffraction analysis, drug loading, drug release, in-vitro anticancer, apoptosis and haemolytic toxicity assay. The surface charge and particle size of the system was found to be -21.0 mV and 234.0 nm respectively. According to the result obtained from morphological assessment (TEM& AFM), the Cur-Gel-CNT was found in nanometric size range and remain in separate form, no adherence was also be obtained in analysis. The release of curcumin from Cur-Gel-CNT was found to be 53.49% after 120 h period of time at 7.4 pH. The release rate of curcumin from Cur-Gel-CNT was found 59.94% and 68.52% in case of 5.5 pH and 4.5 pH after 120 h. As per the result of in-vitro cytotoxicity assay, it was found that, with increasing the concentration of nanocomposites the cell growth was inhibited and the nacrotic cell population was increased from 5.65% to 11.08% obtained from apoptosis assay. The Overall finding obtained from the above analysis it was suggested that the Cur-Gel-CNT system was found in nanometric size range and having superior drug loading efficiency. The in-vitro anticancer assay suggested the anticancer potential of the Cur-Gel-CNT system and the data obtained from apoptosis assay, it was exposed that the system may successfully enhanced the population of nacrotic cells in comparison with control and should be a potential agent for cancer treatment.

***Keywords***: Anticancer; Breast cancer; SWCNTs; Curcumin; Apoptosis.

## Introduction

Carbon nanotubes (CNTs) have played a great role in drug delivery system and CNTs have a amazing properties such as optical, thermal, mechanical and electrical properties with a great researches in pharmaceutical field (Ji *et al.,* 2010; Sinha & Yeow 2005). This nanomaterial is discovered in 1991 and now this is used for controlled drug delivery, controlled release system, targeted drug delivery for cancer therapy and so many areas due its strong affinity for target cells and target tissue (Ijima, 1991; Prato & Kostarelos 2008; Debbage, 2009; Das *et al.,*2009; Sahoo & Labhasetwar 2003). Now a day's cancer is painful disease that enforces severe deaths. Chemotherapy is the one of the great option for the treatment of cancer drug delivery, but everyone knows about the side effects of chemotherapy so with targeted drug delivery system we could reduce the side effects and make our formulation most effective to reduce the size of tumour. Now nanotechnology will work to reduces the side effect and better result of drug delivery system such as CNTs have a greater surface area that will increase the maximum binding efficiency for drug molecule. Curcumin (cur) is a chemical constituent of turmeric and turmeric consists of the dried rhizomes of *Curcuma longa* L. belonging to the family zinziberaceae. Cur have hydrophobic polyphenol compound extract which have strong anticancer activity for breast cancer, colon cancer, colorectal cancer, pancreatic and prostate, head and neck cancer etc, and Cur is most promising anti-tumor drugs, chemo preventive ability (Sahoo *et al.,* 2007), and broad spectrum activity against malignancy (Duvoix *et al.,* 2005; Anand *et al.,*2008; El khoury *et al.,*2019) Cur directly act and inhibit metastasis of various cancer (Karavasili *et al.,* 2019; Komal *et al.,* 2019). Now a day's CNTs used for the drug targeting in cancer and it will show the better results due to their unique physical and chemical properties. CNTs have hollow monolithic structure and a very small size and lager the surface area and also it allows multiple drug attachment inside and outside the wall of CNTs, this system will improve the targeting and it shows also enhanced bioavailability so it will improve the bio distribution of anticancer drug (Wang *et al.,* 2019; Li & Zhang, 2014).

CNTs enters in the cell with different routes like passive diffusion and endocytosis. For the attachment of the drug firstly functionalization of CNTs procedure have done (Kostarelos *et al.,* 2007; Behzadi *et al.,* 2017). Functionalization is important step because the raw CNTs is highly hydrophobic in nature and functionalization incorporate carboxyl groups (due to reaction with strong acid sulphuric acid and nitric acid), after functionalization the drug moieties can be conjugated with covalent bond and non-covalent bond attached with drug on the outer and inner surface of the CNTs. Carboxyl group increases the dispersion of the CNTs in aqueous solution and other solvents (Yuan *et al.,* 2016). After functionalization Cur is attached to the wall of CNTs with modification by gelatin and different chemical groups were also reported to show anti-cancer activity against cancer (Singh *et al.,* 2018; Ward & Courts 1977). Upon hydrolysis of collagen it gives some peptides and protein. Gelatin is the mixture of peptides and protiens and it is also non-toxic in nature (Garg *et al.,* 2016). Upon acid

treatment it will shows the cross-linking property and gelatin is natural biocompatible polymer (Koopman *et al.,* 1994; Van Engeland *et al.,* 1996). The objective of this present research work to develop a Curcumin (Cur) loaded SWCNTs delivery system affixed with geletin and biocompatible cancer-targeted drug delivery system. The chemical structure of gelatin have $NH_2$ group and $NH_2$ attach with carboxylic group of functionalise SWCNTs. This formulation was developed and evaluated for the loading of Cur and release of Cur and increase of solubility of Cur. This developed formulation will be characterized by analytical techniques for its structural, morphological and crystallographic characteristics and evaluated for its ability to encapsulate and release curcumin form the drug delivery system following swelling and degradation mechanisms. The *in vitro* cytotoxicity of the formulation onto MDA-MB-231 human breast cancer cell line will be studied to ensure the proposed application of the delivery system in cancer therapies.

## Materials and Method

Single wall carbon nanotubes (SWCNTs) were purchased from Nanoshel UK Ltd. (Cheshire, CW12 4AB United Kingdom) and curcumin (Mol. Wt. 368.38 g/mol) was purchased from Sigma Aldrich, USA. Sulphuric acid, Nitric acid, Methanol and Glutaraldehyde were procured from Hi-Media (MS) India. Gelatin was purchased from Sigma Aldrich, India. All the other reagents and solvents utilized in the processing and development of nanoplate form was procured from Central Drug House, New Delhi, India.

### Purification process of SWCNTs

For purification of SWCNTs were weighed and kept it into 1M $HNO_3$ (aqueous solution 100ml) for overnight at room temperature (25°C), and vacuum filtered with polycarbonate membrane (0.2 µm) and washed with it deionized water until pH 7 was reached and vacuum dried. The SWCNTs were prolonged sonicated with concentrated sulfuric acid and nitric acid (3:1 40ml, 98 % and 70 %) at 40°C.In this purification and cutting process sonication is the important process because sonication provides an oxidizing acid is that successive attack at the point of damage soon cuts the tube completely because continuous moderate temperatures that open tube ends and that are unable to close. The concentrated sulfuric acid and nitric acid solution mixer add carboxylic group into it. After carboxylation this mixture was washed with 200 ml cold deionized water and filtered it with polycarbonate membrane (0.2 µm) and washed with its deionized water until pH 7 was reached and vacuum dried. Then stored it in well closed container. The method which we are using, this will incorporate carboxyl functional groups on to the side wall of the SWCNTs and it will also improve the biocompatibility and reduce the cytotoxicity (Koopman *et al.,* 1994; Van Engeland *et al.,* 1996).

### Fabrication of Curcumin loaded Gelatin-SWCNTs system

The functionalised SWCNTs was weighed 25 mg and it was dissolved in 10 ml of ethanol to make the suitable dispersion in ultrasonic bath for 10 min. 20 mg of gelatin was weighed and dissolved in 25 ml of water and this solution was heated so that a clear solution is formed. After that the anticancer drug curcumin (Cur) was dissolved in the solution of functionalized SWCNTs and in this solution, gelatin solution was added. Further then glutaraldehyde solution was added drop wise in the Cur loaded SWCNTs-Gelatindispersion and then sonicated it for 30 min (GT Sonic ANTECH). The resultant dispersion curcumin loaded gelatine functionalized MWCNTs (Cur-Gel-CNT) was filtered and washed with de-ionized water and dried at room temperature.

### Characterization & Evaluation parameters

### Fourier transform infrared (FTIR)

0.5 g of final formulation (Cur-Gel-CNT) were taken and crushed with KBr in a mortar and then kept into pellet. These FTIR spectra were taken for the investigation of the chemical interaction of curcumin and the gelatin. FTIR spectra were measured by utilizing FTIR 84005, Shimadzu, with the range 4000–400 $cm^{-1}$.

### Assessment of size and surface charge of Cur-Gel-CNT system

Malvern Zetasizer Nano ZS (Malvern Instrument, UK) with quasi-elastic light scattering was used to determine particle size and zeta potential. 1 mg of formulation (Cur-Gel-CNT) was weighed and dissolved in double distilled water (mili q water) and sonicate it for 5 min. The size of the formulation achieved by triplicates following the dilution of suspension at 25 $C^0$ All measurements were performed in triplicates.

### Morphological features of Cur-Gel-CNT system by AFM

This formulation was further characterized by AFM, the INTEGRA PNL atomic force microscope was taken images. A freshly prepared solution of Cur loaded functionalized SWCNTs were taken and spread uniformly on glass slide and air dried at room temperature and mounted on the microscope scanner. The shape was observed and imaged in noncontact mode with frequency 312 kHz and scan speed 2 Hz.

### TEM (Transmission Electron Microscopy)

JEM-1400 TEM (JEOL, Japan analytical electronic microscope was utilized for assess the morphological characteristics at 80 kV.20uL of the aqueous solution of Cur-Gel-CNT, sample drop was placed on a carbon coated copper TEM grid, and then air dried subsequently analysed.

### X- Ray diffraction (XRD)

The study XRD analysis of vacuum drying the mixture of Cur, Gelatin and Cur loaded geletin-SWCNTs (Cur-Gel-CNT) were determined using with a Siemens D-500 diffractometer (Rigaku Miniflex 600) with Cu-Ka radiation. The scanning angle of diffractometer was set 5° to 40° of 2θ and the measurement were performed at a voltage of 35 kV and 30 mA.

### Determination of loading efficiency of curcumin

In 10 ml of methanol a 10 mg of formulation Cur-Gel-CNT, was dissolved and sonicated, after then centrifuged it for 10 min at 10000 rpm to remove unbounded curcumin, gelatine and SWCNTs in the formulation. Then the supernatant solution was removed and further proceeds for the determination of the concentration of Cur using an RF5301pc spectrofluorometer (Shimadzu, Kyoto, Japan) utilizing a wavelength of 543nm.

**Curcumin release pattern through *in-vitro* release analysis**

In vitro release experiments the release of Cur from formulation (Cur-Gel-CNT) was carried out by dissolving 10 gm of complete dried sample of the Cur-Gel-CNT in 100 mL of phosphate-buffered saline (PBS) solution (0.01 M, pH 7.4) and the solution was divided in 3 portions. The solution was centrifuged at 10000 rpm for 15 min at a pre-determined time(because free Cur is completely insoluble in water) to separate the release of Cur from the final formulation. The curcumin was further extracted in methanol after filtering 2 mL of the solution through a membrane filter with a pore size of 100 nm to separate the released curcumin from the SWCNT-Cur. Subsequently, according to the standard curve of curcumin, which was constructed from the fluorescence intensity of the curcumin as a function of its concentration, the quantities of released curcumin were determined by a spectrofluorometer to calculate the cumulative release rate.

$$\%\ CumulativeRelease = \frac{Amountofcurcuminreleasedinsupernatant}{Totalamountofcurcuminloadedin\ Gelatin - SWCNTs} \times 100$$

***In-vitro* cell cyto-toxicity study by MTT assay**

For assess the cell cytotoxicity of the Cur-Gel-CNT were studied by isolated cells of *in vitro* cultured MDA MB 231 human breast cancer cells. The cells were grown, harvested and seeded in 130 cell per well in the 96-well plates and they were incubated as per previous reporting. The medium which is drug free was replaced by fresh medium which contains different concentration of drug. Then these cells were incubated for 72 h (MDA-MB-231) and maintained physiological conditions at 5% $CO_2$at 37°C (humidified atmosphere).10µL of MTT solution was added to each well and incubated it for 4 h at 37°C, and solubilizing buffer (100µL) was added to each well. There was no staining formed in dead cells and viable cells shows a dark blue/ purple formazan product. The concentration of drug was measured at 570nm by using a micro plate reader. The viability of cell was calculated as a measure of the optical density of treated cells relative to the optical density of the untreated control and dose-dependent response.

**Apoptosis assay**

Culture cells in a 6-well plate at a density of 0.5 x $10^6$ cells/2 ml and incubate in a $CO_2$ incubator overnight at 37°C for 24 hours. Aspirate the spent medium and treat the cells with required concentration of experimental compounds Cur-Gel-CNT and controls, in 2 ml of culture medium and incubate the cells for 24 hours. At the end of the treatment, remove the medium from all the wells and give a PBS wash. Remove the PBS and add 200 µl of trypsin-EDTA solution and incubate at 37°C for 3-4 minutes. Add 2 ml culture medium and harvest the cells directly into 12 x 75 mm polystyrene tubes. Centrifuge the tubes for five minutes at 300 x g at 25°C. Carefully decant the supernatant. Wash the cells twice with PBS. Decant the PBS completely. Add 5 µl of FITC Annexin V. Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark. Add 5 µl of PI and 400 µl of 1X Binding Buffer to each tube and vortex gently. Analyze by flow cytometry immediately after addition of PI (Koopman *et al.,* 1994; Van Engeland *et al.,* 1996).

**Cell Cycle assay**

In a 6-well plate at a density of 2 × $10^5$cells/2 ml cultured cells were incubated in a $CO_2$ incubator overnight at 37°C for 24 hours. The cells were treated with spent medium and required concentration of required chemical compound Cur-Gel-CNT, and controls in 2 ml of culture medium and incubated the cells for 24 hours. The medium was removed from the wells and washed with the PBS buffer, after washing PBS was removed and added 200 µl of trypsin-EDTA solution and incubate it for 3-4 min at 37°C. 2 ml of culture medium was added and the cells were harvested directly into 12 × 75 mm polystyrene tubes. The tubes were centrifuged for five minutes at 300 × g at 25°C. Carefully decant the supernatant and washed with PBS and decant the PBS completely. Then fixed with 1 ml cold 70% ethanol and added cell pellet drop wise while vortexing and cells were fix properly and minimize clumping. Incubate for 30 min. -20°C freezer. Pellet cells at higher speed compared to live cells for 5 minutes, aspirate the supernatant being careful not to lose the pellet. Ethanol fixed cells required higher centrifugal speeds to pellet compared to unfixed cells since they become more buoyant upon fixation and washed twice with PBS (Garg *et al.,* 2016).

**Stability study of curcumin**

To estimate the stability profiling of synthesized formulation Cur-Gel-CNT was placed with PBS (0.01 M, pH 7.4), for duration of 1 at various temperature range 4°C, 25°C and 45°C, to observe the stability of the system. The stability was estimated by color changes, turbidity, particle size of nano-formulation and % drug loading efficacy.

**Statistical analysis**

Statistical evaluation was repeated about 3 times and attained by t -test $P < 0.05$ and it is important. The investigational conclusion given as mean ± SD (number of experiments). All the experiment was repeated about 3 times.

## Result & Discussion

**FTIR spectroscopic study**

SWCNTs are generally insoluble in water and poor dispersibility in biosystem so that we proceed it for functionalization (kept it into conc. $H_2SO_4$ and $HNO_3$), carboxylation takes places and these characteristic peaks of carboxyl(C=O) confirm with 1650 $cm^{-1}$ in the infrared spectrum and this peak confirm the carboxylation. The functionalise SWCNTs shows the characteristic peaks of -COO groups at 1635.69 $cm^{-1}$ on the surface of the Cur-Gel-CNT and this will show the successful oxidative modification. The curcumin peaks in the system shows C-O stretching peaks at 1118.75 $cm^{-1}$, $CH_3$ bending 1375.29 $cm^{-1}$, $CH_2$ bending 1431.23 $cm^{-1}$, benzene ring bending vibration 1508.38 $cm^{-1}$, C=O stretching1627.97 $cm^{-1}$, C-H aromatic stretching vibration 3018.70 $cm^{-1}$. The overall findings suggested that, the identification of the carboxylated MWCNTs have characteristic peaks of C=O stretching at 1650 $cm^{-1}$ and the peak obtained at 3250 $cm^{-1}$ suggested the presence of N-H moiety in the structure, which justify the covalent attachment of the MWCNTs with curcumin via C=O & N-H functionality group (Figure 1).

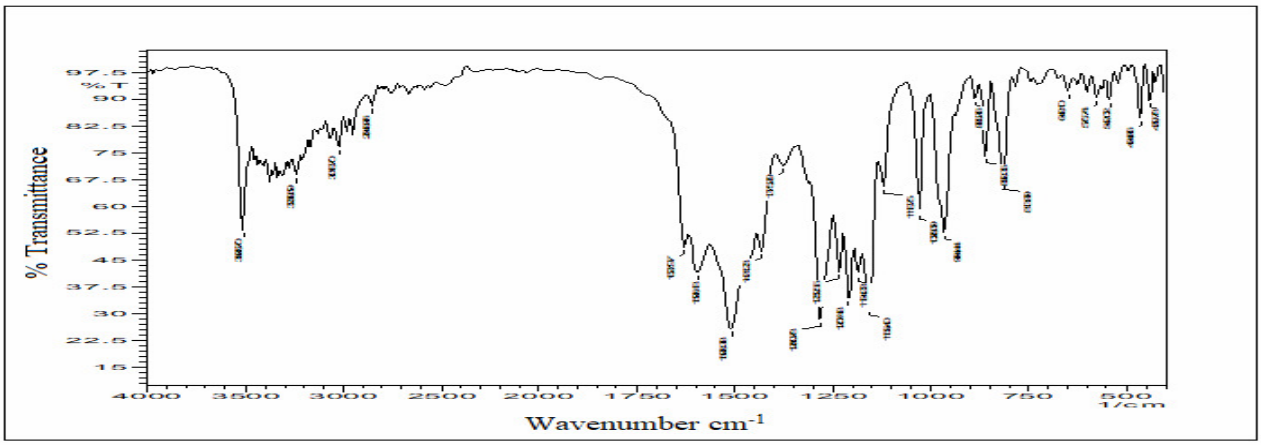


**Fig. 1a:** FTIR spectrum of Native Curcumin.

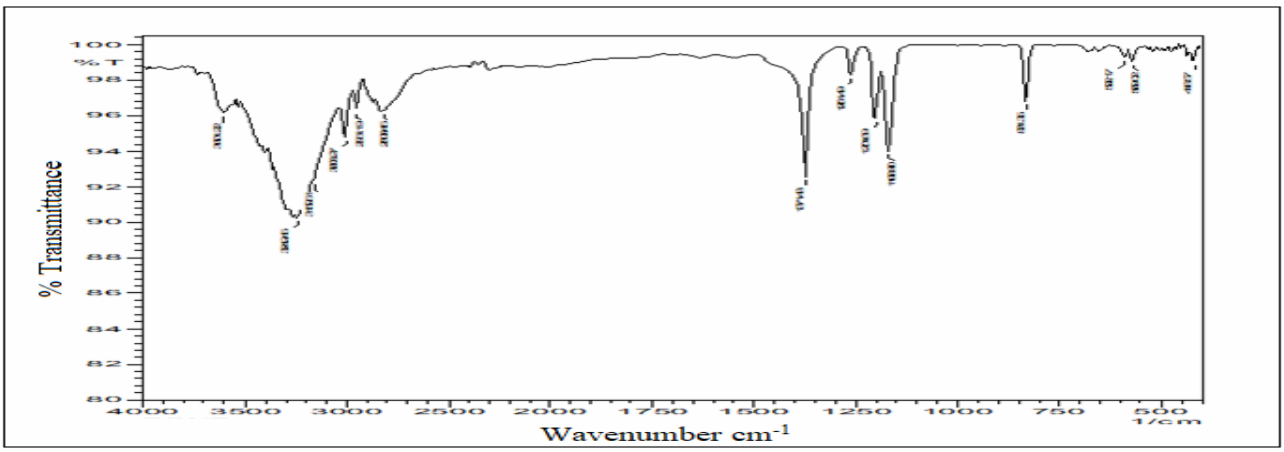


**Fig. 1b:** FTIR spectrum of Functionalize SWCNTs.

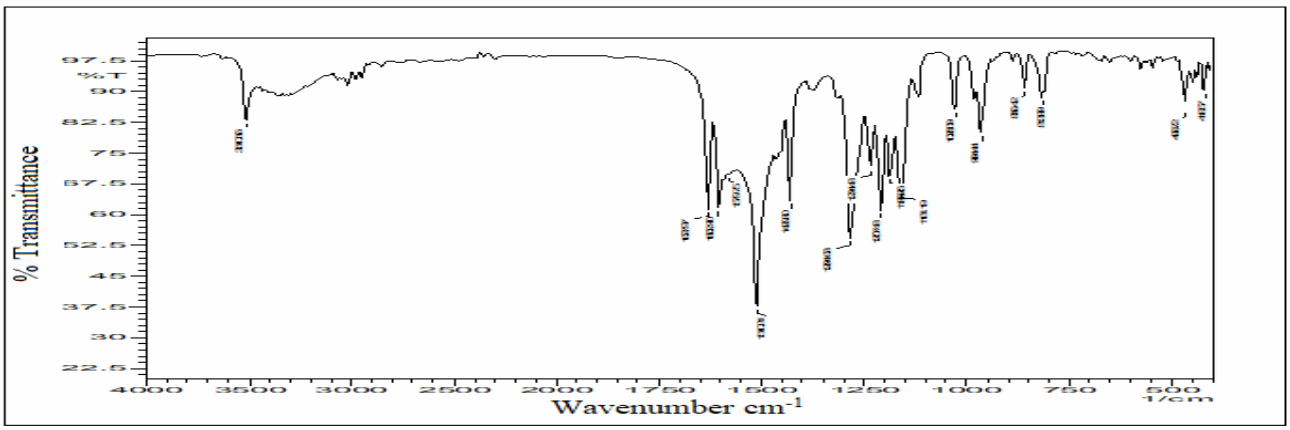


**Fig. 1c:** FTIR spectrum of formulated Cur-Gel-CNT conjugate.

**Particle size and zeta potential measurements**

For the determination of the different variation of surface charge of modification of each step were determine through zeta potential study. Treatment with conc. $H_2SO_4$ and $HNO_3$ introduced carboxyl group and hydroxyl group, this surface modification shows negative zeta potential (-20.5 mV) and this suggest that formulation may be suitable for in vivo administration. When oxidised SWCNTs reacts with the curcumin and gelatin this interaction shows zeta potential values becomes positively charge (30.4 mV) (Figure 2), this shows that the cationic charged formulation exhibit endocytotic cellular uptake by negatively charged.

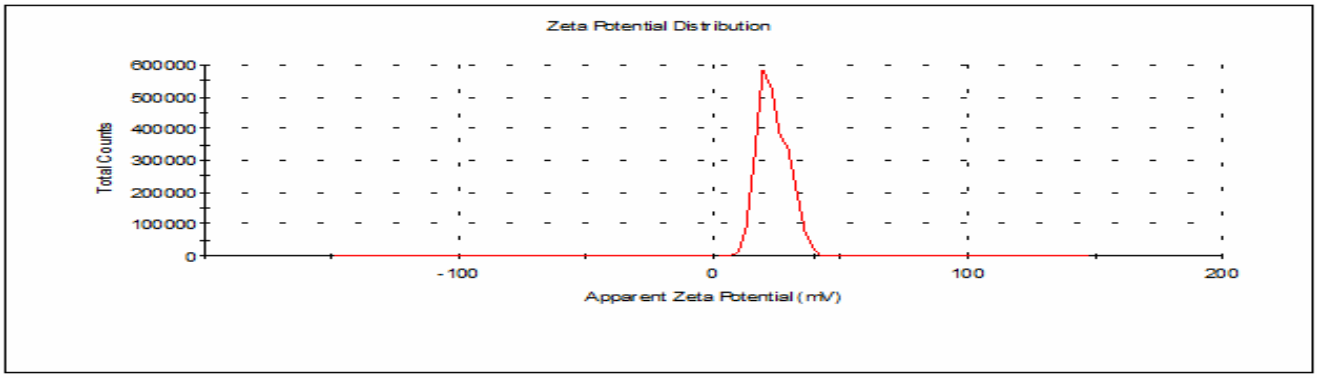


**Fig. 2:** Zeta potential of curcumin conjugated gelatine functionalized SWCNTs (Cur-Gel-CNT).

Zeta potential data investigation shows the gelatin attach with SWCNTs with $NH_2$ bonds and this will show π-π stacking. The particle size of prepared Cur-Gel-CNT system was also be monitored and the size was found to be 193.47 nm, which suggested that the formulated Cur-Gel-CNT system was in nano level range. The Cur-Gel-CNT formulation have numerous concentration levels of SWCNTs and curcumin and the whole system enhanced, which is shown by the enhancing the concentration the size of nano system enhanced. The % of loading efficiency was affected by above phenomenon for SWCNTs attached with curcumin. Higher the size range of system the % EE was reduced. The final optimizes Cur-Gel-CNTs formulation having size range was 234.00 nm (Figure 3), having the drug loading proficiency was 94.15% (Table 1).

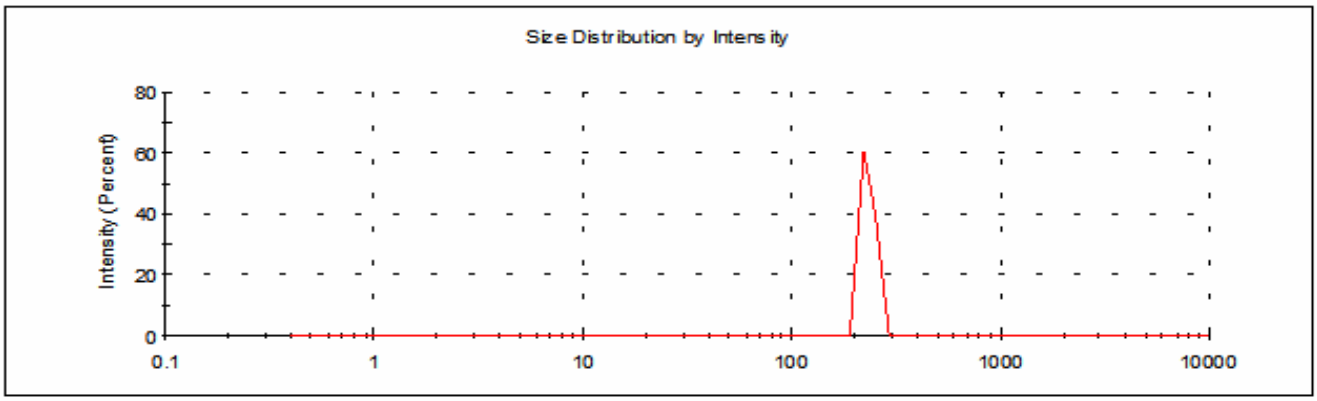


**Fig. 3:** Particle size of curcumin conjugated gelatine functionalized SWCNTs (Cur-Gel-CNT).

**Table 1 :** Optimization parameters for formulation of Cur-Gel-CNT.

| S. No. | Concentration of ingredients | | Particle size (nm) | % drug loading |
|---|---|---|---|---|
| | SWCNTs (mg) | Curcumin (mg) | | |
| 1 | 10 mg | 10 mg | 179.41 ± 0.51 nm | 84.12 ± 0.50 % |
| 2 | 10 mg | 20 mg | 182.54 ± 0.80 nm | 83.98 ± 0.65 % |
| 3 | 20 mg | 10 mg | 188.14 ± 1.12 nm | 91.21 ± 1.15 % |
| 4 | 20 mg | 20 mg | 193.47 ± 1.20 nm | 94.15 ± 1.55 % |
| 5 | 30 mg | 10 mg | 247.12 ± 1.55 nm | 81.50 ± 1.65 % |
| 6 | 30 mg | 20 mg | 262.31 ± 1.98 nm | 76.45 ± 1.75 % |

**Morphological assessment by Transmission Electron Microscope (TEM) and Atomic force microscope (AFM)**

For the investigation of the structure of Cur-Gel-CNT was observed through analysis with a microscope technique in which a beam of electrons was transmitted on specimen to from an image. In the image of Cur-Gel-CNT light colored chains were shows on the side wall of SWCNTs. High resolution TEM image shows average diameter of formulation 35-40 nm (Figure 4a). The polymeric modification in formulation (Cur-Gel-CNT) conforms with the AFM analysis with surface roughness (Figure 4b). Reaction with concentrated acid the surface of SWCNTs shows rougher surface because the wall of SWCNTs gets defected due to acid treatment, and when we further treat it with polymer and Cur so the image of formulation shows the smoother surface due to polymeric interaction and cross linking with each other molecule. TEM & AFM is carried out for morphological examination of formulation and the images for curcumin loaded gelatine functionalized SWCNTs shows small aggregates bundle and the Cur-Gel-CNT formulation shows the uniform distribution and found in separated pattern. This will improve the dispersibility of formulation (Figure 4).

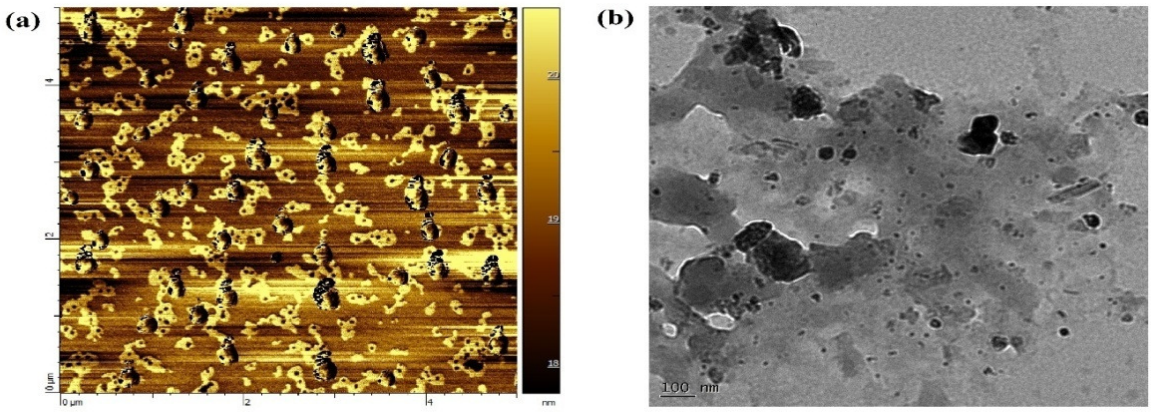


**Fig. 4(a):** TEM and (b)AFM of curcumin conjugated gelatine functionalized SWCNTs (Cur-Gel-CNT).

## X-Ray Differaction analysis

The XRD patterns were measured to estimate the physical state of formulation i.e. amorphous molecular state and crystalline states and its distribution and to determine the release of Cur from synthesized formulation (Cur-Gel-CNT) and related to its molecular form. The XRD patterns of native curcumin exhibited characteristic high intensity diffraction peaks at 2θ values for amorphous state was 6.6, 8.96, 13.838, 16.622, 18.299, 25.323, 28.21 and suggested crystalline state peaks at various 2θ values, this shows the native curcumin is in highly crystalline form (Figure 5a). The synthesized formulation Cur-Gel-CNT was showed 2θ values 9.32, 17.748, 18.51, 22.94, 23.97, 31.945 and 46.04 and in this the sharp characteristic peaks of curcumin was optimized at 9.32, 18.51 and 22.94-25.56, which indicates the curcumin conjugated with SWCNTs and also shows the complete attachment with SWCNTs (Figure 5b).

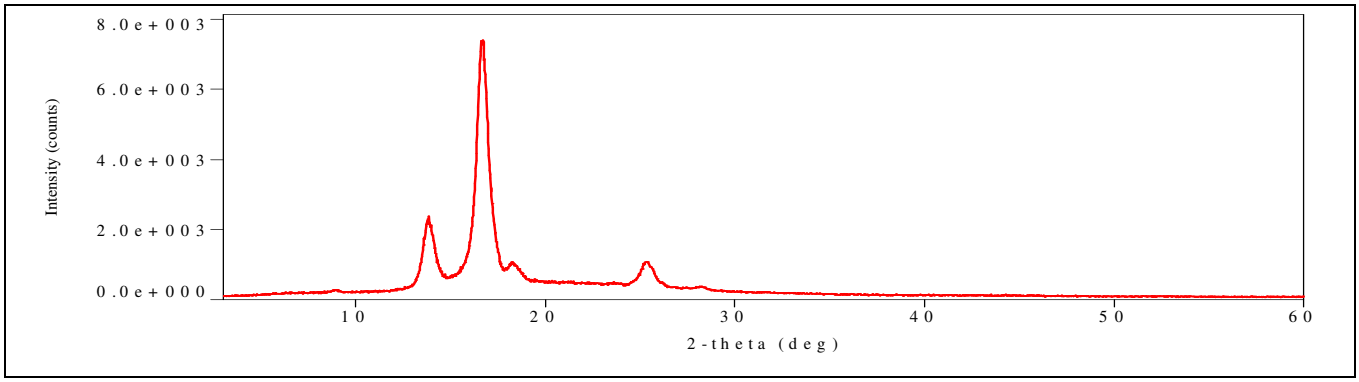


**Fig. 5a:** X-Ray differactogram of curcumin

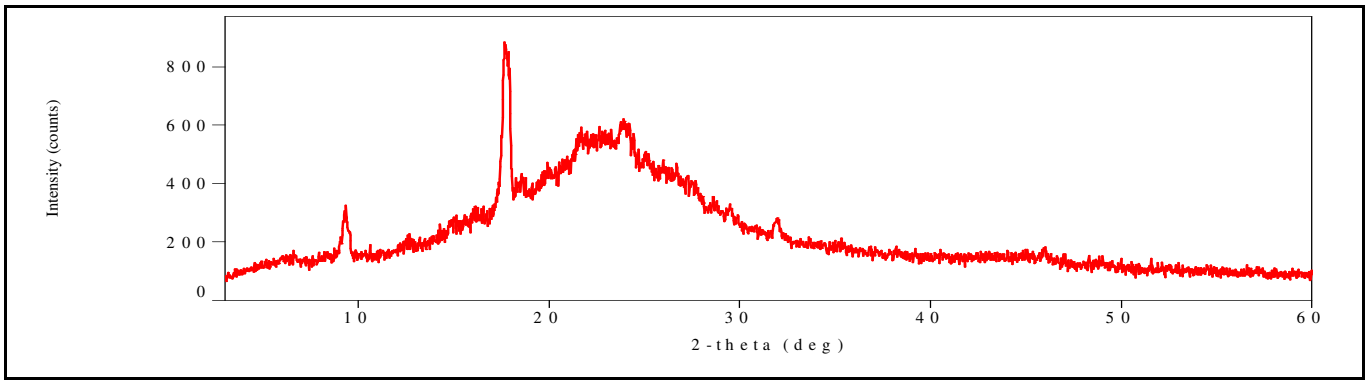


**Fig. 5b:** X-Ray differactogram of curcumin conjugated gelatine functionalized SWCNTs (Cur-Gel-CNT).

## Determination of loading efficiency of curcumin

The Cur-Gel-CNT show high drug loading efficiency but slow drug release due to attachment with gelatin shows slow drug loading efficiency and fast drug release. In the formulation Cur was attached through π-π stacking interaction with the side wall of the formulation. The loading efficiency was estimated for the formulation94.15±1.55%.In the experiment curcumin dissolved in methanol exhibit in molecular state with the surface of SWCNTs. Due to small in the size the SWCNTs have a larger surface area and gelatin also have a big structure and it is biopolymer which is generally prepared by thermal denaturation of collagen and curcumin contains two benzene ring and conjugate with ethylenic linkage, when the solution kept into ultrasonic bath, the energy of ultrasonic bath curcumin attached with the

SWCNTs because of NH2 group linkage (gelatin have NH2 group both side so in one side nh2 attach with the SWCNTs and another side with curcumin)and hydrogen bond form between the carboxyl group of curcumin. Varderwaals force enhance the loading of curcumin in SWCNTs because both are hydrophobic in nature. During the preparation of the the conjugation that is observed that the sonication time and energy plays a great role and effects on the drug loading efficiency.

**Drug release phenomenon**

The identification of drug release characteristics (*in-vitro*) of Cur in the formulation Cur-Gel-CNT was determined through drug release study and drug release is critical activity. The result showed that the release of Cur in formulation to be 82.10 % after 8h period of time at 7.4 pH and in 5.5 pH the release was found to be 90.14 % and in 4.5 pH the release was found to be 94.23 % at 8h. The cumulative release rate of curcumin exhibited downward trend and this can be explained by the degradation of Cur. The formulation of Cur-Gel-CNT, the release of curcumin was found to be 53.49 % after 120 h period of time at 7.4 pH. The release rate of curcumin from Cur-Gel-CNT was found the 59.94 % and 68.52 % in case of 5.5 pH and 4.5 pH after 120 h (Figure 6).The acidic pH shows higher release rate and basic pH shows slow release rate because of breaking tendency of covalent bond between Cur-Gel-CNT. The release rate was higher in acidic pH then basic pH 7.4.

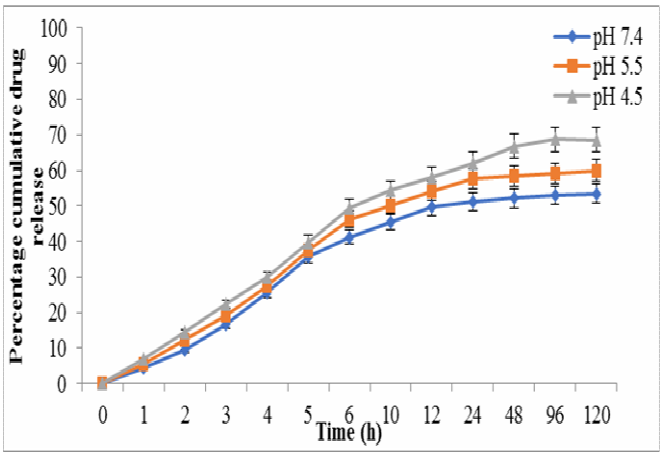


**Fig. 6 :** Drug release characteristics of Cur-Gel-CNT at various pH (7.4, 5.5 & 4.5) range.

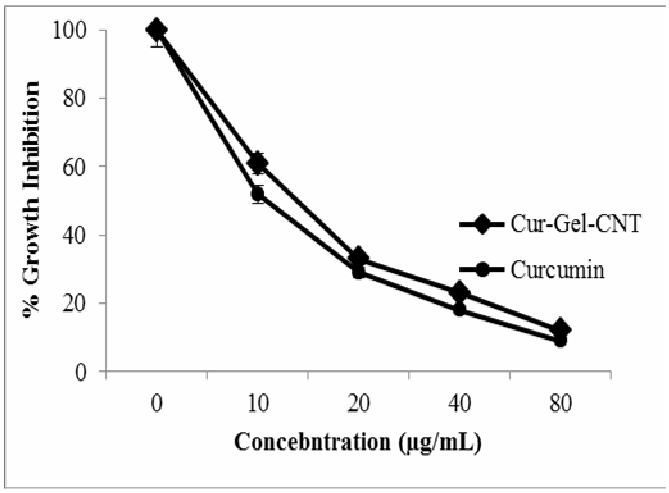


**Fig. 7 :** In-vitro cell cytotoxicity profiling by MTT assay of Cur-Gel-CNT against MDA-MB-231 cellline.

**Cytotoxicity test**

To know the viability of cell studied by MTT assay and the assay were carried out to determine the cytotoxicity effects of Cur-Gel-CNT. This analysis was performed with different concentration of human breast cancer cellline MDA-MB-231. The analysis shows the result that the growth was inhibited with increasing the concentration of Cur-Gel-CNT. The data was concluded according to the above study that the Cur-Gel-CNT have potency to decrease the growth of cancer cell and also inhibit the cell viability at the different concentration range (Figure 7). This mechanism shows the selective mode of action can be ascribed to enhance the drug release under acidic conditions, according with the in vitro release studies. This modification Cur-Gel-CNT will be considered as appropriate candidates for anticancer drug delivery.

**Apoptosis**

To estimate the apoptotic profiling of the synthesized compound Cur-Gel-CNT, using IC50 concentration (25µg/ml) compared with control utilizing FACS based Annexin V-PI expression study on the human breast cancer cell line (MDA-MB-231). The observations suggested us that the test compound Cur-Gel-CNT, induces the significant late and early apoptosis in human breast cancer cells (Table 2). The compound Cur-Gel-CNT may have therapeutic potential against human breast cancer and further preclinical studies have to be done to confirm the mechanism of action on MDA MB 231 cells (Figure 8&9).

**Table 2:** Annexin V/PI expression study in MDA MB 231 cell line.

| Quadrant | %Necrotic cells | %Late apoptotic cells | %Viable cells | % Early apoptotic cells |
|---|---|---|---|---|
| Label | UL | UR | LL | LR |
| Cell Control | 11.08 | 0.01 | 88.04 | 0.77 |
| Cur-Gel-CNT | 5.65 | 38.04 | 37.1 | 19.21 |

**Note:** Table showing the % of cells of undergone Apoptosis in untreated and test compound, Cur-Gel-CNT (with IC50 concentration) treated MDA MB 231 cells.

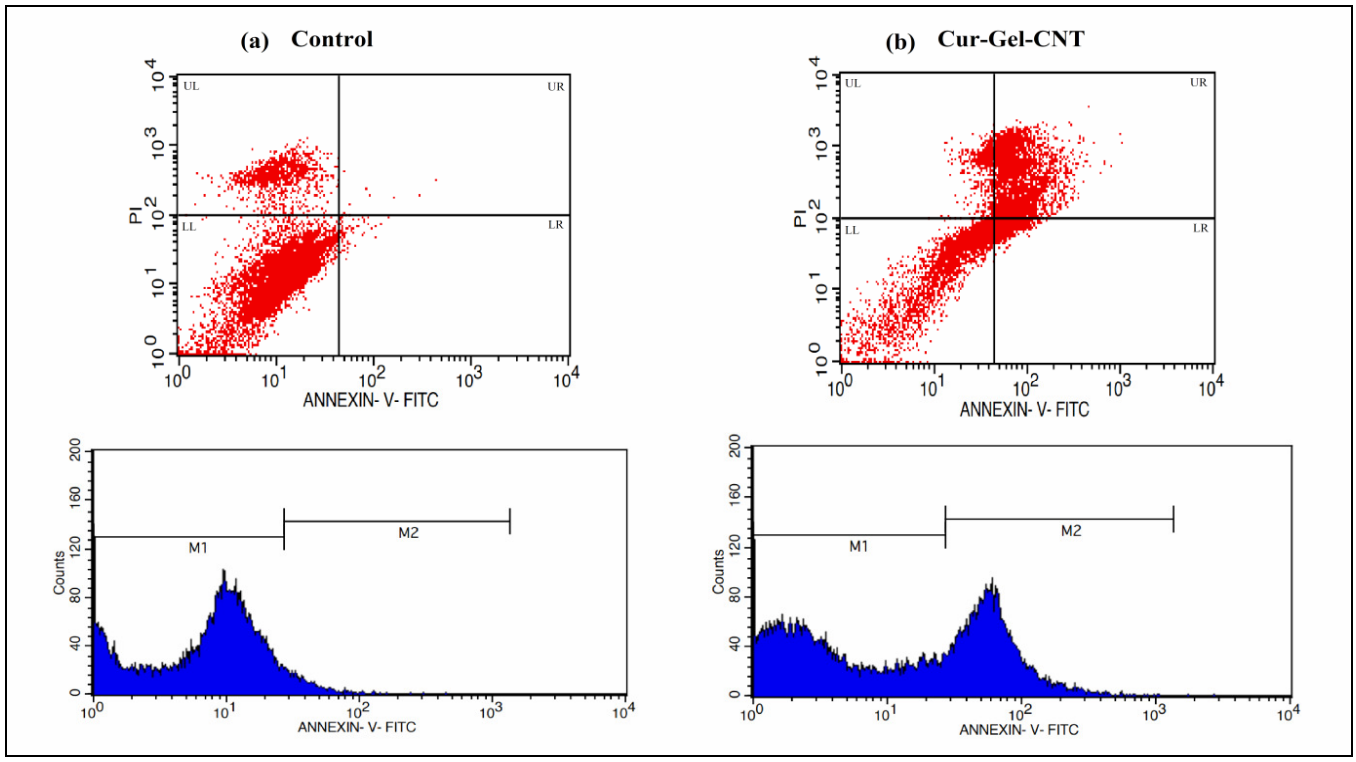


**Fig. 8:** Annexin V/PI expression Study of the Cur-Gel-CNT against the MDA MB 231 Cell line. Qudrangular plot representing the Annexin V/PI expression in MDA MB 231 cells upon culturing in the presence and absence of test compound, Cur-Gel-CNT. Analysis was done by using BD FACScalibur, Cell Quest Pro Software (Version: 6.0). Annexin V-FITC - Primary Marker, PI- Propidium Iodide (Secondary fluorescence Marker). **Abbr.:** UL – Upper left: % of Necrotic Cells; UR - Upper right: % Late Apoptotic Cells; LL- Lower left: % Viable Cells; LR- Lower right: % of Early apoptotic cells.

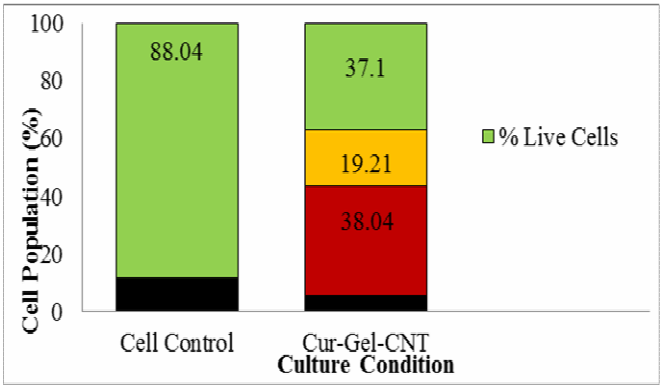


**Fig. 9:** Bar graph showing the % of apoptotic and necrotic cells.

### Cell cycle analysis

The formulation Cur-Gel-CNT, showed significant cell inhibition, with the selected IC50 concentration of 25 µg/ml against the MDA MB 231 cell lines. The cell cycle study conducted by Flow Cytometry to check the stages of cell cycle arrest and the obtained results by Flow Cytometry depicted in Table 3 and Figure 10&11. The cells treated with Cur-Gel-CNT, with the $IC_{50}$ concentration showing high % of cells at G2/M phase and moderate S phase arrest, when compared to untreated cells. Cur-Gel-CNT exhibiting prominent G2/M Cell Cycle phase arrest MDA MB 231 cells.

**Table 3 :** Cell cycle analysis: Percentage of cells in different cell cycle stages vs MDA MB 231.

| S. No. | Cell Cycle stage | Cell Control | Cur-Gel-CNT |
|---|---|---|---|
| 1 | Sub G0/G1 | 0.6 | 2.45 |
| 2 | G0/G1 | 74.49 | 59.15 |
| 3 | S | 6.11 | 9.7 |
| 4 | G2/M | 18.94 | 27.26 |

**Note:** Table showing the % of cells get arrested in the different stages of their life cycle. In Sub G0/G1 phase (Apoptotic phase), **0.6% and 2.45%** of cells get arrested in Untreated and test compound Cur-Gel-CNT respectively. In G0/G1 phase (Growth Phase), **74.49% and 59.15%** of cells get arrested in Untreated and test compound Cur-Gel-CNT respectively. In S phase (synthetic phase), **6.11% and 9.7%** of cells get arrested in Untreated and test compound Cur-Gel-CNT respectively. On the other hand, in G2/M phase, **18.94% and 27.26%** of cells get arrested in Untreated and test compound Cur-Gel-CNT respectively.

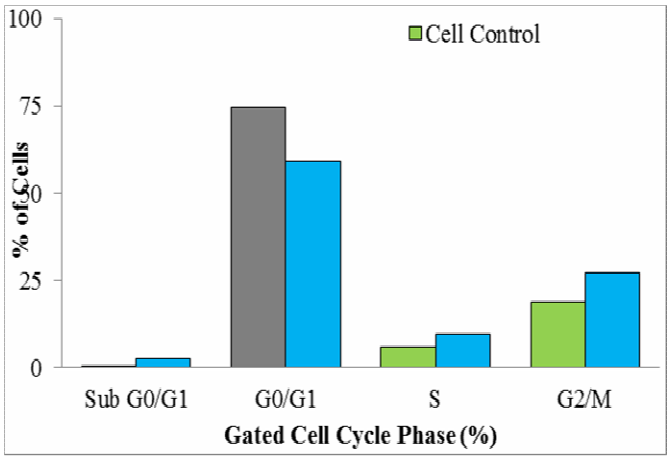


**Fig. 10:** Overlay showing the % of cells get arrested in the different stages of their life cycle.

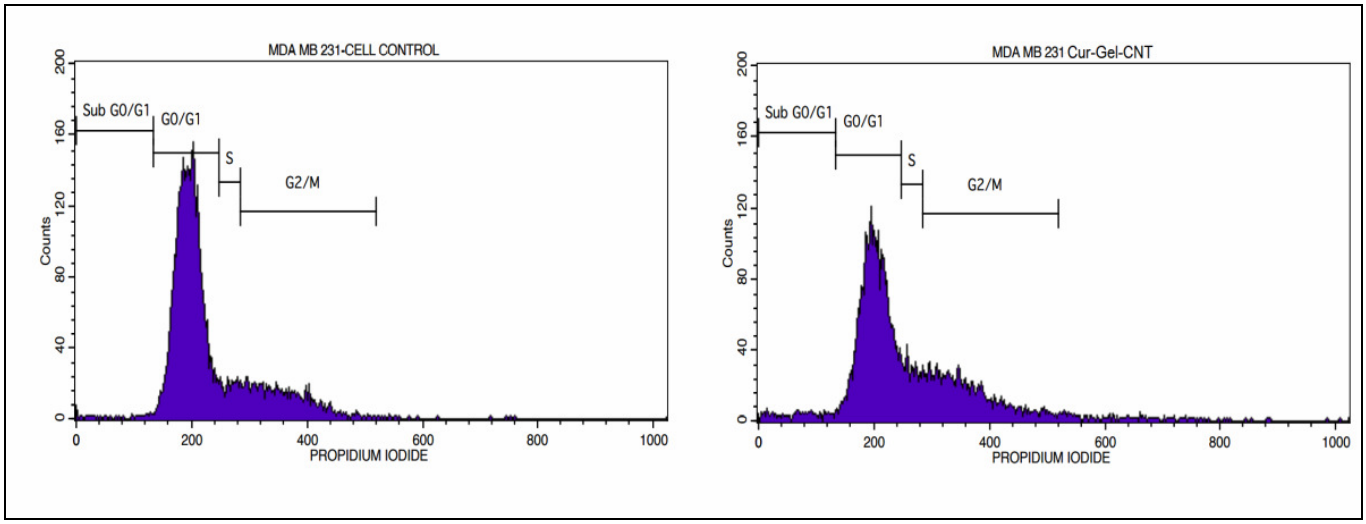


**Fig. 11:** Flow cytometric histograms showing the phases of cell cycle distribution in the MDA MB 231 cell line treated with the given test compounds viz, Cur-Gel-CNT with $IC_{50}$ values compared to the control.

### Stability study of curcumin

To estimate the stability profiling of synthesized formulation Cur-Gel-CNT was placed with PBS (0.01 M, pH 7.4), for duration of 1 at various temperature range 4°C, 25°C and 45°C,to observe the stability of the system. The stability was estimated by color changes, turbidity, particle size of nano-formulation and % drug loading efficacy. The Cur-Gel-CNT system was found, not change in color, clear dispersion, stable particle size and drug loading after 15 days and 30 days period at 4°C, 25°C and 45°C. These data reveal that the Cur-Gel-CNT system may stable all the temperature range in one duration of one-month interval.

## Conclusion

The Cur-Gel-CNT conjugate with curcumin (anticancer drug) via amide linkage utilize with gelatin as linker (also acts as copolymer) for effective targeting against human breast cancer cell lines MDA-MB-231.The Cur-Gel-CNT conjugation with curcumin was evaluated with various parameter such as particle size, drug loading, drug release, TEM, AFM, apoptosis, cell cycle. The overall finding obtained from the above analysis it was suggested that the Cur-Gel-CNT system was found in nano size range and having the entrapment efficiency shows higher entrapment efficiency. The drug release characteristics also shows higher drug release in acidic pH then basic pH and the characteristic also suggested that the release of curcumin from Cur-Gel-CNT found in higher release manner. The in vitro MTT assay (anticancer assay) shows the anticancer potential of the Cur-Gel-CNT system and the data obtained from apoptosis and MTT assay, it was revealed that the system may effectively increase thre population of nacrotic cell in comparison with control and it should be potential drug for the treatment of cancer.

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