Frontiers in Health Informatics
ISSN-Online: 2676-7104
www.healthinformaticsjournal.com
2024; Vol 13: Issue 8
Open Access

Development And Characterization Of Folic Acid Conjugated Multi Walled Carbon Nanotubes

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Cite this paper as: Jitendra Kayat, Pooja Tiwari, Rituraj Singh Chundawat, Neeraj Sharma, Ruchi Jain (2024). Development And Characterization Of Folic Acid Conjugated Multi Walled Carbon Nanotubes. *Frontiers in Health Informatics*, *Vol.13, No.8*, 6606-6615

Abstract

The development of folic acid conjugated multi wall carbon nanotubes (FA-MWCNTs) allowed for the regulated and less toxic distribution of methotrecate (MTX). MWCNTs were functionalized one after the other to create MTX/FA-MWCNTs. In phosphate buffer solutions (PBS) with a pH of 7.4, MTX was physically loaded onto virgin MWCNTs and FA-MWCNTs. The entrapment efficiency, in vitro release, hemolytic toxicity, and ex vivo tests on the lung epithelial cancer cell line "A-549" were assessed. MTX was effectively incorporated into both the pristine and FA-MWCNT formulations, with the FA-MWCNTS formulation exhibiting the maximum trapping of MTX at 90.3 ± 1.8%. The study examined the in-vitro release of MTX from MTX/FA-MWCNTs at 7.4 (PBS), exhibiting a quicker initial release followed by a sustained release. Furthermore, it was discovered that, when applied to the lung epithelial cancer cell line "A-549," MTX/FA-MWCNTs was more cytotoxic and less hemolytic than free MTX.

Key words: - Methotrexate, Folic acid, Functionalization, Dihydrofolate Reductase.

INTRODUCTION

Among the materials discovered in the last 30 years, the properties of carbon nanotubes have attracted intense interest from the scientific community as well (Harris, 1999). Finding novel and efficient drug delivery methods is a crucial problem that will always be of interest. The general goal of a drug delivery system is to enhance a medication molecule's pharmacological and therapeutic characteristics (Theresa, Pieter, 2004) The possibility to use f-CNT as vehicles for the delivery of tiny therapeutic molecules is provided by their capacity to enter cells (Nadine, Theodore, Paul, Hongjie, 2004). CNT can be creatively created by rolling up several graphene sheets to form concentric cylinders (multi-walled CNT) or by rolling up a single layer of graphene sheet (single-walled CNT) (Sumio, Toshinari, 1993). Commercially available as-produced CNT with varying degrees of purity and distinct structural features is available for both SW and MW (Bethune *et al.*, 1993). A number of biological applications, including drug administration, seem particularly promising for soluble CNT, which may be prepared thanks to the development of effective methods for chemical modification of CNT (Alberto, Kostas, Charalambos, Maurizio, 2005; Henghe, Bruce, 2007). For the modification of CNT, two

functionalization techniques are commonly used. Strong acids can be used to oxidize CNT, which reduces their length and produces carboxylic groups that make them more soluble in aqueous solutions (Kostarelos, Lacerda, Partidos, Prato, Bianco, 2005). As an alternative, CNTs become soluble in water through addition reactions to their tips and external walls (Liu *et al.*, 1998). For carbon nanotubes to be considered biocompatible, they must be soluble in physiological solutions. Furthermore, peptides, proteins, nucleic acids, and other medicinal agents can be connected to a broad range of active molecules via functionalized carbon nanotubes (f-CNT).

The folic acid (FA) antagonist methotrexate (MTX) binds to the dihydrofolate reductase enzyme to stop the synthesis of purine and pyrimidine bases, serine, and methionine amino acids (Dimitrios, Nikos, Vasilios, Maurizio, 2003). It is used to treat a variety of malignancies, including tumors of the nose, breast, and lung. Lowering the medication dosage of MTX will lessen its negative effects. It is frequently given intravenously or intramuscularly and is typically used in higher doses for the treatment of cancer than are necessary for other conditions. Because of the drug's significant systemic toxicity, its usefulness is restricted. According to multiple reports, methotrexate-containing targeted anti-cancer delivery systems have drug loading capacities ranging from 12% to 80%, depending on the components and preparation techniques used (Aranya, Yatindra, Linda, Dane, 1985; Vodovozova, Kuznetsova, Gaenko, Molotkovsky, 2007). Allotropes of carbon, carbon nanotubes have a length to diameter ratio of more than one million in their nanostructure. Their hollow cylinders can have diameters as small as 0.7 nm and lengths of several millimeters, with a hexagonal network of carbon atoms woven throughout (Annabelle, 2004).

In this work, we produced methotrexate-loaded folic acid conjugated multiwalled carbon nanotubes (MTX/FA-MWCNTs) as a means of increasing the availability of methotrexate. Fourier transform infrared (FT-IR) spectroscopy and transmission electron microscopy (TEM) were used to further characterize the produced MTX/FA-MWCNTs. Ultimately, the proposed formulation was subjected to entrapment efficiency, in-vitro release profile, and cytotoxicity experiments.

MATERIAL AND METHODS

Multi-walled carbon nanotubes (MWCNTs) produced by chemical vapour deposition (CVD) with purity of 99.3 %, diameter × length 110-170 nm ×5-9 µm and melting point 3652-3697° C, was purchased from Platonic Nanotech Private Limited, Jharkhand, India. Methotrexate was purchased from Triveni Interchem Pvt. Ltd., Gujrat, India. All reagent like Ethylene diamine, N,N-dimethyl formamide, Tetrahydrofyran(THF), Dimethylsulphoxide (DMSO), N-Hydroxysuccinimide (NHS) and folic acid (FA) were purchased from commercial supplier and used as received.

Purification of carbon nanotubes

MWCNTs purification based on the concepts of metallic impurity dissolving by acids and selective oxidation, where carbonaceous impurities oxidize more quickly than CNTs (Peng-Xiang, Chang, Hui-Ming, 2003). Catalytic and amorphous impurities were eliminated from the unpurified MWCNTs by acid treatment. A certain amount of unpurified MWCNTs was obtained and then placed into strong hydrochloric acid for five hours while being stirred magnetically. The mixture was then filtered using a 0.45 µm polytetrafluoroethylene (PTFE)

filter (Sigma Aldrich, USA). In order to eliminate the amorphous carbon, acid-purified MWCNTs were placed in an oven and kept there for 30 minutes at 530°C (Jinyong, Yafei, 2006; Prashant, Raksha, Narendra, 2012).

Cutting and Carboxylation of purified MWCNTs

The type of polymer to be reinforced determines which chemical group ought to be bonded to the nanotubes. Several authors (Goyanes, Rubiolo, Salazar, Jimeno, Corcuera, Mondragon, 2007) have suggested that the presence of carboxylic acid groups on the surface of carbon nanotubes (CNTs) is a common path towards accomplishing this goal because these groups can be the source of a wide range of chemical reactions.

The concentrated H2SO4 and HNO3 (3:1) ratio was used to oxidize the purified MWCNTs in a sonication tube for 15 minutes. After sonication, the prepared suspension was put into a round-bottom flask (RBF) and magnetically stirred for four and twelve hours at 60 ± 2°C. It was then filtered and cleaned with deionized water. Lastly, the black solid residue was vacuum-dried for an entire night at room temperature (RT) (Jinyong, Yafei, 2006; Prashant, Raksha, Narendra, 2012).

Acylation and amidation of carboxylated MWCNTs

For 24 hours, carboxylated MWCNTs were continuously stirred at 70 ± 2°C while 30 mL of thionyl chloride (SOCl2) and dimethyl formamide (DMF) were added in a 20:1 ratio. To remove extra thionyl chloride, the resultant suspension was filtered and five times treated with anhydrous tetrahydrofuran (THF). An oven with a vacuum was used to dry the residual solid. For two days, at 100 ± 2°C, 10 mL of ethylene diamine solution (EDA) was reacted with 20 mg of acyl-chlorinated MWCNTs. To get rid of extra diamine, MWCNTs were cooled to room temperature and then five times in ethanol. Ultimately, the black solid residue was vacuum-dried for an entire night at room temperature (Jinyong, Yafei, 2006; Prashant, Raksha, Narendra, 2012).

Conjugation of folic acid to amine modified MWCNTs

A beaker containing 25 ml of methanol was filled with known quantity of amine-modified MWCNTs, and 500 milligrams of folic acid was added to the mixture. After processing the reaction for five days at room temperature while stirring continuously, acetone was added to produce a yellow precipitate. Filtered and dried, folate conjugated MWCNTs (f-MWNCTs) were analyzed using techniques such as FT-IR, H-NMR, and XRD (Neelesh, Jain, 2013; Neelesh, Jain, 2015; Rakesh, Tathagata, Abhishek, Alok, Bhudev, Narendra, 2008).

Loading of drug (Methotrexate) in MWCNTs

Evaluation of FA conjugated MWCNTs' drug-holding and drug-release capabilities in release media was part of their characterization process. To help in drug encapsulation, twenty milligrams of MTX and ten milligrams of f-MWCNTs (2:1 ratio) dispersed in phosphate buffer saline (pH=7.4) were combined, and the mixture was agitated overnight. The drug-loaded f-MWCNTs were separated from the solution by ultracentrifugation after encapsulation. By measuring the amount of MTX in supernatant with a UV spectrophotometer (Shimadzu 1601, Japan), the amount of MTX entrapped in f-MWCNTs based systems was determined independently. A similar process was used with pristine MWCNTs (Jitendra, Neelesh, Virendra, Narendra, 2016).

***In vitro* Drug release studies**

Under physiological conditions (PBS; pH 704), the *in vitro* release of medication from two different formulations (pristine MWCNTs and f-MWCNTs) was determined. For release investigations, the dialysis membrane (MWCO; 2000 Da) was chosen. After inserting five milligrams of the formulation into the dialysis sac and sealing it from the outside, it was suspended in 100 milliliters of aqueous receptor release medium right away. The *in vitro* drug release experiment was performed in the receptor compartment at 37±0.5°C with continual stirring under tight sink conditions. At each of the prearranged intervals, one milliliter of the sample was taken out and replaced with an equivalent volume of new medium. Drug was measured using a spectrophotometer (UV/Vis Shimadzu 1601, Japan) at 258 nm after the proper dilutions.

Hemolytic Toxicity

In Hi-Anticlot blood collection vials (Hi media, India), whole human blood was drawn. The tube was centrifuged at 3000 rpm for 15 minutes using Remi equipment from India, resulting in the separation of red blood cells (RBCs) at the bottom. Normal saline (0.9% w/v) was used to wash the RBCs until a clear, colorless supernatant was obtained above the cell mass. Re-suspended in normal saline solution were the cells (Mishra, Gupta, Jain, 2010). The RBC suspension was supplemented with deionized water and normal saline with the consideration of 100% and no hemolysis, respectively. After mixing pristine MWCNTs, MTX/FA-MWCNTs, and free MTX with normal saline and letting them stand for a couple of hours while shaking, the mixture was centrifuged for fifteen minutes at 3000 RPM. The resulting supernatant was then diluted with an equivalent volume of normal saline and absorbance was measured at 480 nm (UV/vis 1601, Shimadzu, Japan) (DOX equivalent). This equation was used to compute the percentage of hemolysis:

$$\text{Hemolysis } \% = \frac{(\text{Abs}-\text{Abs}_0)}{(\text{Abs}_{100}-\text{Abs}_0)} \times 100$$

where samples' absorbance is represented by the symbols Abs, Abs_0, and Abs_{100}, which stand for samples' absorbance at 0% hemolysis and 100% hemolysis, respectively..

Cytotoxicity studies

An environment of 37 ± 0.5°C, humidified with 5% CO2, was used to culture the lung epithelial cancer cell line A-549 in RPMI-1640 medium. The medium was supplemented with 10% heat-inactivated fetal calf serum (FCS), 2 mM glutamine, and antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin) (Agrawal, Gupta, Asthana, Jian, 2009). A non-radioactive colorimetric assay based on 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was utilized in the investigation to measure the inhibition of cell growth. By incubating A-549 cells with the free MTX and MTX/FA-MWCNTs formulation in vitro, cytotoxicity was assessed. The A-549 cells were cultivated to 80% confluence in 37 ± 0.5°C humidified incubator with 5% CO2 environment using RPMI-1640 media supplemented with 10% fetal calf serum, 2 mM glutamine, 50 IU/mL penicillin, and 50 µg/mL streptomycin. In 96 tissue culture plates with a flat bottom, A-549 cells were planted at a density of 2×10^5 cells/mL and incubated for a full day. The cultivated cells were subjected to sequential treatments of free MTX and MTX/FA-MWCNTs suspension at concentrations of 01–10 µg/mL, and were then incubated for 24 hours in a controlled environment. Then, each well was filled with MTT solution dissolved in phosphate buffer solution (PBS; pH 7.4), and the wells were incubated

for 8 hours at 37 ± 0.5°C to allow the viable cells to convert the MTT to purple formazan crystals. Using the following equation, cell viability was assessed in an Elisa plate reader at 570 nm. For every sample in the MTT experiment, three replicates were read, and the mean value was utilized to determine the final result, which was significant ($p < 0.05$).

Stability studies

For five weeks, the suspension of MTX/FA-MWCNTs was kept in tightly sealed amber and colorless glass vials at four degrees Celsius, room temperature (25 degrees Celsius), and thirty-five degrees Celsius in an oven with a thermostat. The suspension was periodically checked for changes in precipitation, crystallization, color, consistency, and turbidity. The acquired data was utilized to analyze any degradation, both chemical and physical, and to recommend the necessary storage conditions. Monitoring drug release from the MTX/FA-MWCNTs suspension following storage under various circumstances allowed for the determination of drug leakage as well. To do this, a weekly sample of 2 mL was taken, suitably diluted, and subjected to an HPLC method of analysis. This process was repeated every week for a maximum of five weeks.

RESULT AND DISCUSSION

The MWCNTs were acquired from Jharkhand, India's Platonic Nanotech Private Limited. There is only one peak in the purified pristine MWCNTs' FT-IR spectra (FIGURE 1), indicating that there are no impurities present. Folic acid was coupled with amine-terminated MWCNTs (FIGURE 2). The aromatic carbon-hydrogen bending peak, as well as the aromatic carbon=carbon bending and stretching peak, are visible in the FT-IR spectra of FA conjugated MWCNTs (f-MWCNTs).

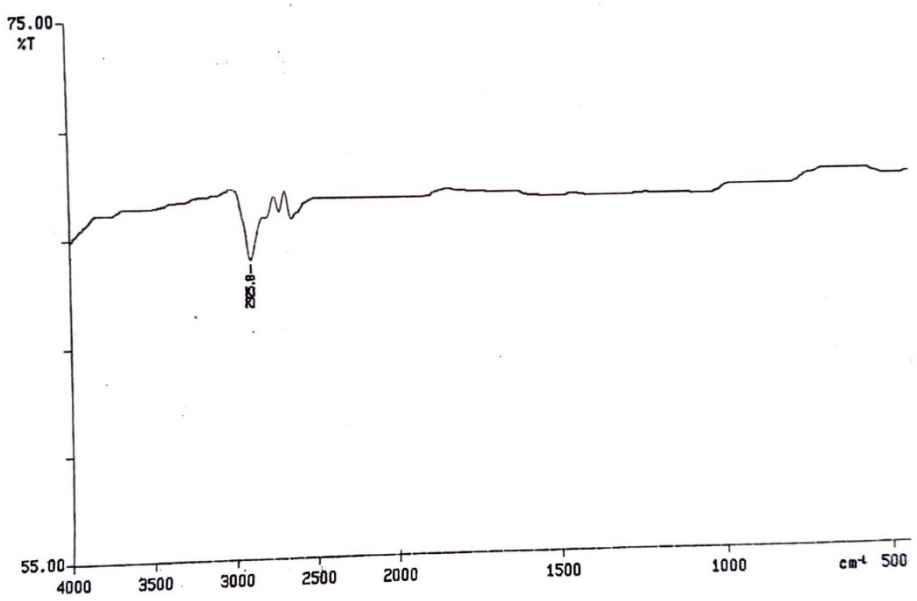

FIGURE 1:- FT-IR spectrum of Pristine MWCNTs

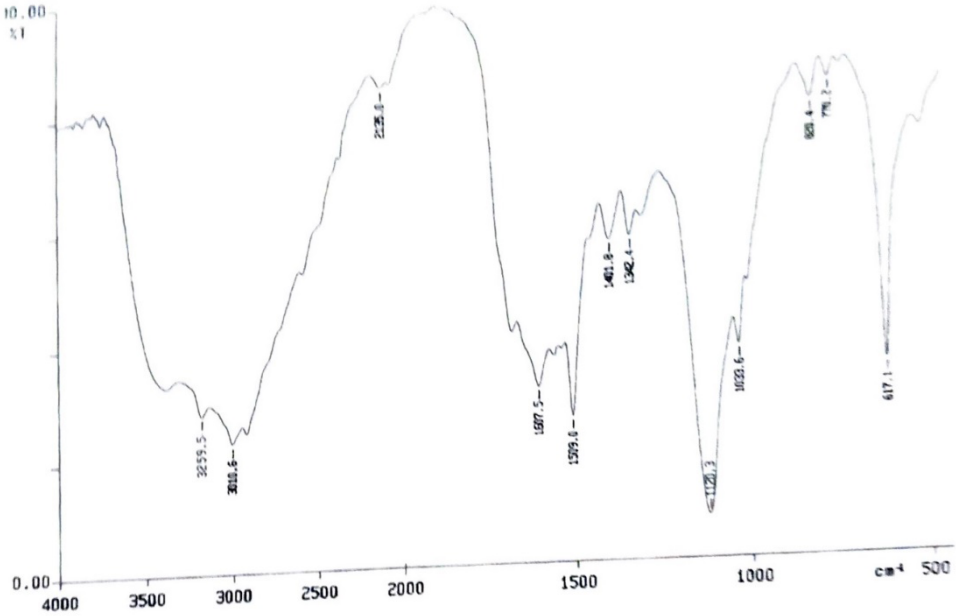

FIGURE 2:- FT-IR spectrum of Folic Acid Conjugated MWCNTs

Using the equilibrium dialysis approach, methotrexate (MTX) was loaded into pristine and conjugated folic acid-containing MWCNTs. The drug loading experiment was carried out in a phosphate buffer saline pH 7.4 dispersion medium at room temperature. For folic acid conjugated MWCNTs (FA-MWCNTs), a higher drug loading of 90.3 ± 1.8% was noted. On the other hand, pure MWCNTs showed lower drug loading, at 65.4 ±2.1% (FIGURE 3).

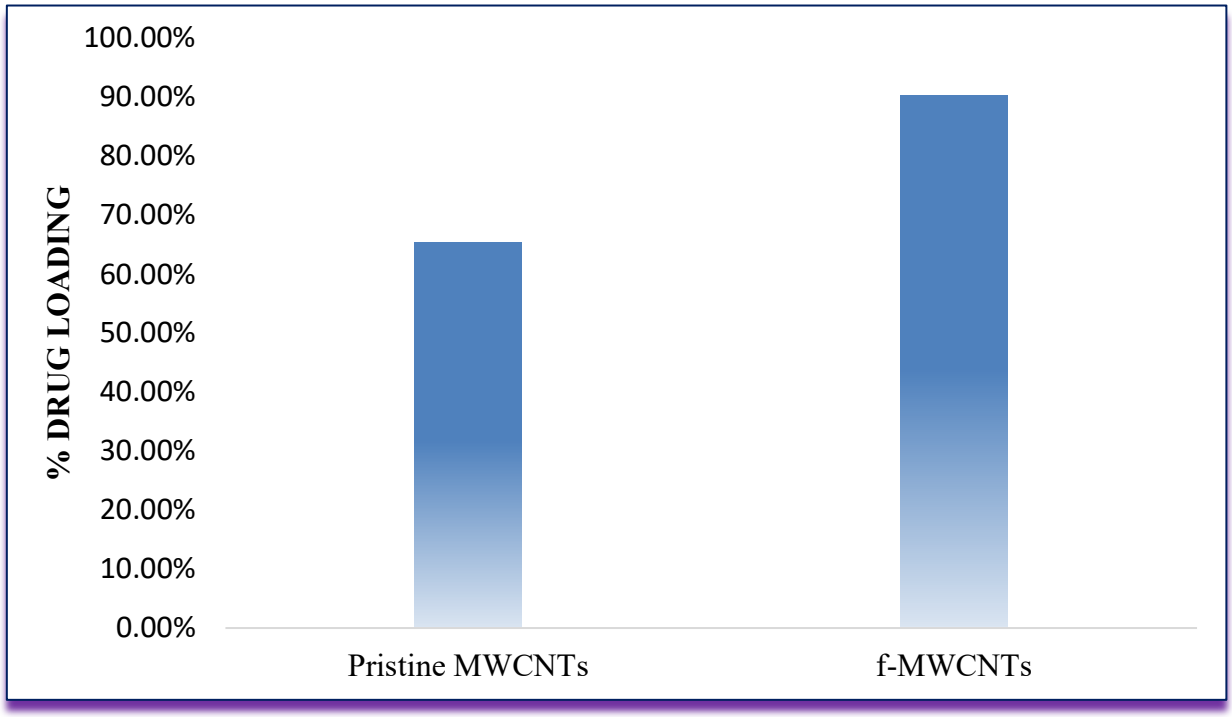

FIGURE 3:- Drug loading efficiency (n=3)

Phosphate buffer saline pH 7.4 was used as the receptor media in the equilibrium dialysis method, which was used to measure the release of MTX from folic acid linked MWCNTs and pristine MWCNTs. In a 24-hour period, the drug release from pristine MWCNTs was observed to be 62.34±1.8%, whereas folic acid conjugated MWCNTs released 53.67±1.3% (FIGURE

4).

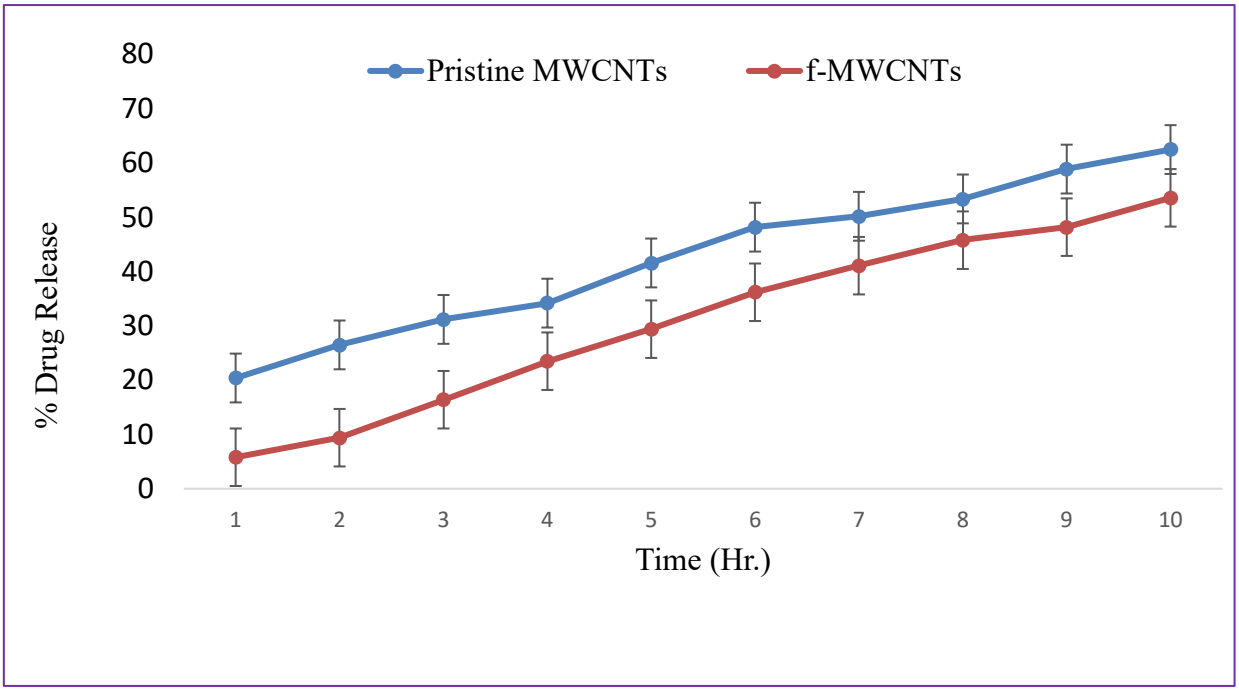

FIGURE 4:- % Drug Release of MTX in Pristine & Folic acid attached MWCNTs (n=3)

The use of pristine MWCNTs as a drug delivery device was limited due to their hemolytic toxicity and drug-loaded composition. Due to the decreased availability of free medicines, the folic acid affixed to the surface of MWCNTs significantly decreased the percentage of hemolysis of RBCs that was achievable.

The MTT test was used to evaluate the cytotoxicity of both free MTX and MTX/FA-MWCNTs on A-549 cells at equivalent dosages ranging from 1 to 10 µg/mL. The results unequivocally demonstrated that MTX/FA-MWCNTs inhibited A-549 cells in a dose-dependent manner and were noticeably more cytotoxic at higher concentrations. Since MTX/FA-MWCNTs target specific ligands, they exhibited a highly cytotoxic response. FIGURE 5 shows the uptake of methotrexate by the lung epithelial cancer cell line in our investigations, which revealed dose-dependent uptake.

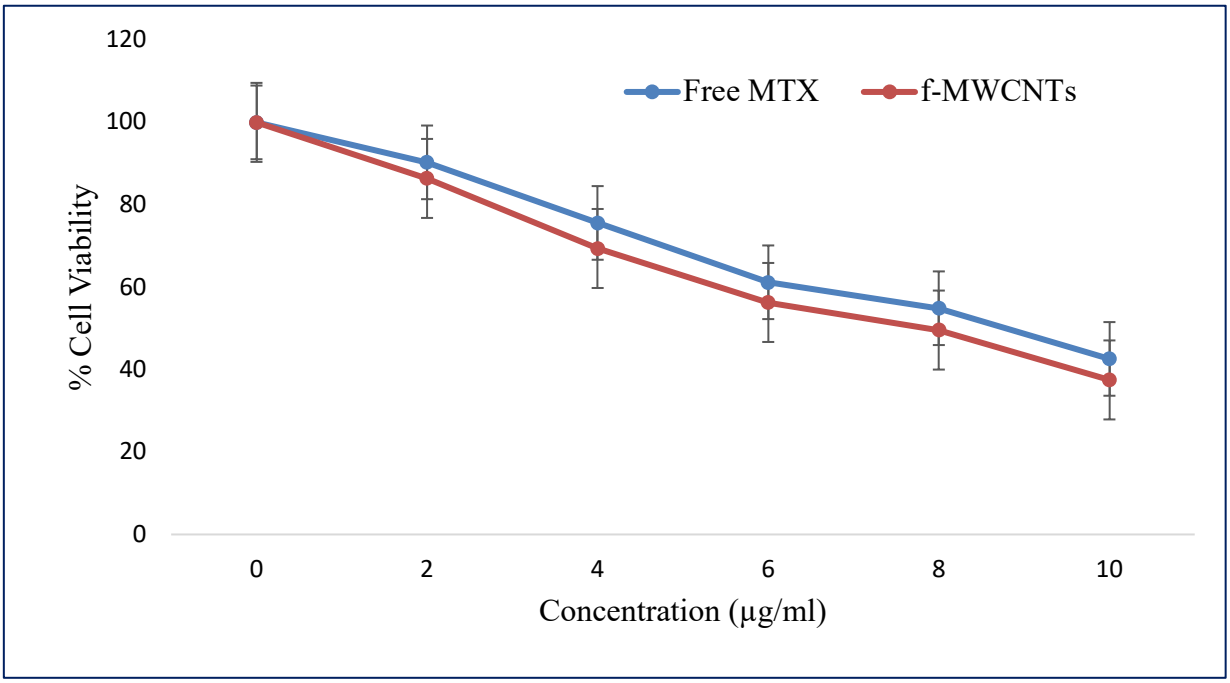

FIGURE 5:- % cell viability assay of free MTX & MTX/FA-MWCNTs formulation (n=3)

CONCLUSION

CNTs are a novel type of carriers that can be used to deliver medications in a target- and site-specific way. Because of their unique mechanical, chemical, and physical characteristics, CNTs are an effective biological carrier for anticancer medications. Owing to their distinct chemistry and hexagonal carbon atom arrangement, carbon nanotubes (CNTs) offer multiple sites for covalent and noncovalent functionalization with therapeutically active molecules. This suggests that CNTs may be used as nanocarriers to deliver therapeutic agents to specific cancer cell targets. These functionalized CNTs have a strong inclination to cross cell membranes through independent or dependent mechanisms on endocytosis. The formulation of MTX-loaded folic acid-MWCNTs demonstrated effective MTX release to the intended site with an enhanced therapeutic margin of safety, it may be inferred.

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