Journal of Chemical Health Risks

www.jchr.org

JCHR (2023) 13(4s), 329-335 | ISSN:2251-6727

Development and Assessment of Carbon Nanotube-Curcumin Modified Nanoconjugate System for Breast Cancer Targeting

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(Received: 02 September 2023 Revised: 14 October Accepted: 07 November)

KEYWORDS
Anticancer, Breast Cancer, CNTs, Curcumin, Cytotoxicity.

ABSTRACT:
The objective of the current research was to formulate SWCNTs anchoredcurcumin utilizing adipic acid dihydrazide (ADH) via carbodiimide conjugation for efficient targeting against human breast cancer celllines (MDA-MB-231). The CNT-C 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 -19.5 mV and 178.21 nm respectively. According to the result obtained from morphologicalassessment (TEM), the 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 CNT was found to be 58.36% after 120 h period of time at 7.4 pH. The release rate of curcumin from CNT was found 62.35% and 69.58% 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 3.25% to 18.98% obtained from apoptosis assay. The Overall finding obtained from the above analysisit was suggested that the 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 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.

INTRODUCTION

In present global cancer scenario, herbal drugs obtained from plant sources have been proved effective in reducing the suffering of patients affected by various types of cancers. Synthetic drugs, surgeries and other treatment options fail and finally when the life expectancy is only 5 to 7 weeks or months are given, an individual for last hope searches natural remedies to sustain their life. In most of the cancer cases, patients suffer from immense pain which is intolerable and to get well soon many cancer patients are inclined plant-based remedies to restrict further growth and propagation of cancer to other parts of the body, restricting cancer in the affected body organs only and also improve the quality of life. The use of synthetic drugs and their analogues even though have been proved to be effective in some cancer casesbut their tremendous side effects along with other common side effects associated with their usage have impelled the scientists to accomplish an alternative approachwith improved patient survival in cancer drug discovery program.

Herbal Medicine is the used mostly for the treatment of various diseases and now at the new scenario everyone prefers herbal drug due its less side effect in thebody and due to its less toxic effect in the body, we used multiple approaches are very common in single treatment especially in very critical disease state and life- threatening condition of the patients. Herbs have different meanings but in the present context (“herb” as

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www.jchr.org
JCHR (2023) 13(4s), 329-335 | ISSN:2251-6727

used in herbal medicines is also known as botanical medicine in our country or in Europe and other advanced countries as phytotherapy or phytomedicines) it refers 'a plant or plant part used in the treatment of common to chronic diseases for therapeutic properties to maintain or improve health'. Herbal remedies popularly known as botanicals or phyto medicine or phytotherapeuticals are the preparations made from different parts of the plant. An herb can be a leaf, a root, seed or fruit or bark or stem used for its medicinal properties.

Cancer (word coined by the father of medicine, Hippocrates, a Greek physician) is a renegade system of growth that originates within human biosystem. It involves uncontrolled and unregulated cell growth inside the body system [Jaggi,2005]. Medically it is termed as malignant neoplasm (new cells/tissues with defects or abnormal growth or division of cells/tissues). It is generally found in anypart of the body and sign and symptoms are different at particular sight. Malignant neoplasm or cancerous cells or tissues are not self-controlled in its growth and often invade adjacent cells or tissues and some times travel to distant parts of the body.Oldest description of cancer was discovered in Egyptian mommies 3000 BC back and till date researchers are finding an appropriate cancer treatment therapy [Morton, 1997].

The global burden of cancer is increasing tremendously. This is largely because of aging and growth of world population along with cancer causing behavior like chewing tobacco, smoking particularly in economic developing countries. Based on the GLOBOCAN (Global Cancer Network) 2008 estimates, about 12.7 millioncancer cases and 7.6 million cancer deaths are estimated to have occurred in 2008; of these, 56% of the cases and 64% of the deaths occurred in the economically developing world. Breast cancer is the most frequently diagnosed cancer and the leading cause of cancer death among females, accounting for 23% of the total cancer cases and 14% of the cancer deaths. Lung cancer is the leading cancer site in males, comprising 17% of the total new cancer cases and 23% of the total cancer deaths.

Nanotechnology (nano = very small 1×10^{-9}m /1×10^{-3}µm and technology = knowledge of techniques) is defined as the emerging and diversified fields in which ultra-fine particles are transformed by controlling on an atomic or molecular level into human friendly myriad of applications. Nano composition has a smaller size range and greater the effects of different treatment due to a larger surface area and greater bioavailability. *Curcuma Longa* is a potential folklore medicinal plant of Indian origin, belonging to the family Zingiberaceae. It is widely used in Ayurveda and other Indian folk medicine for the treatment of various diseases. Carbon nanotubes (CNTs) are the greatest research material now a days and it is also known as buckytubes because they are spherically structured carbon allotropes, are cylindrical carbon molecules with unique properties and that make them potentially useful in a wide variety of applications. CNTs include applications in nano-electronics, optics, and materials applications. Many of research on carbon nanotubes (CNTs) has focused on exploiting the wide range of outstanding mechanical, electronic, and thermal properties of these materials [Kamboj, 2001 and Padh, 2000]. Many of these motivating and unique properties can only be realized once the CNTs are combined into more complex assemblies [Jaggi, 2005]. Functionalization is a common technique to incorporate SWCNTs into different assemblies. Functionalization is done through different chemical process or acid treatment. From chemical treatment enables chemical covalent bonding between the SWCNTs and the material of interest [Morton, et al., 1997; Jemal, et al., 2011].

MATERIAL AND METHOD

Materials

Single divider carbon nanotubes were purchased from Nanoshel UK Ltd. (Cheshire, CW12 4AB United Kingdom) and curcumin (Mol. Wt. 368.38 g/mol) was purchasedfrom Sigma Aldrich, USA. Sulphuric acid, Nitric acid, Methanol and Glutaraldehyde were obtained from Hi-Media (MS) India. ADH was purchased from Sigma Aldrich,India. The wide scope of different reagents and solvents utilized in the taking care of and improvement of nanoplatforms was acquired from Central Drug House, New Delhi, India.

Processing of SWCNTs

Cutting, Purification and Oxidation of SWCNTs

For dealing with the SWCNTs, we followed a practically identical strategy as portrayed as of now [Villa et al.,2008]. In an analysis, the away from of SWCNTs was saved until further notice (short-term) with 1M HNO_3 at room temperature (25°C). By then, they were filtered, washed with sanitized water until a pH 7 was refined, ultimately vacuum dried. By and by following an uncovered strategy, the as- cleaned SWCNTs were first treated with a mix of concentrated sulfuric and nitricacids (3:1, 40 mL), and this mix was then sonicated for 3 h at 40°C in a ultrasonic shower to present carboxylic acid gatherings on the SWCNT surfaces [Liu et al., 1998]. It is worth to make reference to here that despite the fact that hydrogenation [Pekker et al.,2001] and fluorination [Mickelson et al., 1998] functionalization techniques have additionally been accounted for in the writing, carboxylation [Chen et al., 1998] is a basic, effective and appropriate for the current work since it tends to be covalently clung to shape ester or amide linkages [Garg et al., 2012]. In the interminable stockpile of carboxylation, the mix was

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JCHR (2023) 13(4s), 329-335 | ISSN:2251-6727

washed with 200 mL of cold refined water and thereafter isolated through a polycarbonate channel paper. The development, which was functionalized SWCNTs, was totally washed with refined water until a pH of 7 was reached finally it was vacuum dried at 80°C for 4 h. The as- functionalized SWCNTs were taken care of in an invulnerable compartment.

Synthesis of SWCNTurcumin

Actuated SWCNTs (20mg) were broken up in ethanol (10mL) to make the sensible dissipating. In this scattering, the ADH (100mg) was added with relentless blending for the association of NH2 moiety of ADH with carboxylated SWCNTs for the game plan of SWCNTs-ADH-NH2. After then, the medication scattering (curcumin: ethanol with 20mg: 10mL) was incorporated drop astute path into started (enactment of the plan) SWCNTs-ADH-NH2. This cycle is finished using an ultrasonic shower (GT Sonic ANTECH) for 10min, by then rotating disappeared to dryness to get SWCNTs complexed with curcumin. The complex was then sonicated for 15 min, and the suspension was consequently ultrasonicated (100 w, on numerous occasions) using a JV92-II ultrasonic cell aggravation structure (Ninbo Scientz Biotechnology CO., Ltd., Zhejiang, China). The ensuing suspension was centrifuged at 5000rpm for 10min to wipe out the excess curcumin and insoluble SWCNTs, by then the supernatants were lyophilized to make the last meaning of SWCNTs-ADH-CUR and debilitated to 10mL going before use, aside from whenever communicated something different.

Characterization parameters of Nanoconjugate

FTIR spectroscopic evaluation

Fourier-transform infrared spectroscopy (FTIR) is a procedure used to get an infrared range of absorption or emission of a solid, liquid and gas. A FTIR spectrometer all the while gathers high-resolution spectral data over a wide otherworldly reach. This presents a critical preferred position over a dispersive spectrometer, which estimates force over a thin scope of frequencies all at once. For the affirmation of Fourier Transform Infrared Spectroscopy (FTIR), the Samples(2 mg) were blended in with 0.5–1 g of KBr. This mix was painstakingly beat in a mortar, and sometime later kept into the humble pellet. FTIR range was recorded on (FTIR 84005, Shimadzu) Thermo Nicolet spectrometer in the show up at 4000–400 cm−1.

Size distribution and zeta potential determination

Molecule Size and zeta potential were made plans to use a Malvern Zeta-sizer Nano ZS (Malvern instrument, UK) considering semi-flexible light dispersing. Rapidly 1 mg/ml of Carbon nanotubes course of action was set up in twofold refined water (miliq water) and sonicate for 5 minutes in water shower (Thermo legitimate, Genesys10uv looking at). Particle size assessment was acted in arrangements of three of every comparable instrument at 25 °C using the above show. All assessments were acted in arrangements of three.

Morphological assessment by transmission electron microscopy (TEM)

Transmission electron microscopy (TEM) is a microscopy procedure wherein a light emission is communicated through an example to shape a picture. The example is regularly a ultrathin area under 100 nm thick or a suspension on a network. A pictureis framed from the association of the electrons with the example as the bar is sent through the example. The picture is then amplified and centered onto an imaginggadget, for example, a fluorescent screen, a layer of photographic film, or a sensor,for example, a scintillator connected to a charge-coupled gadget. Transmission electron magnifying lens are equipped for imaging at an altogether higher resolution than light magnifying microscope, attributable to the more modest de Broglie frequency of electrons. This empowers the instrument to catch fine detail—even as little as a solitary segment of molecules, which is a great many occasions more modest than a resolvable item found in a light magnifying lens. Transmission electron microscopy is a significant logical technique in the physical, substance and natural sciences. TEMs discover application in disease exploration, virology, and materials science just as contamination, nanotechnology and semiconductor research, yet in addition in different fields, for example, fossil science and palynology. TEM imaging test was performed to recognize the presence of SWCNT aggregates. After a drop of an example suspension was determined to the TEM systems and dried, the TEM examination was performed with a JEM-1400 TEM (JEOL, Japan) working at 80kV.

X-ray diffraction (XRD) characterization

The XRD examples of vacuum drying the mix of curcumin, SWCNTs, andADH in methanol; the strong curcumin; and the genuine mix of curcumin, ADH, and SWCNTs, were made plans to use a Siemens D-500 diffractometer (Rigaku Miniflex 600) with Cu Ka radiation (k¼1.5405A˚). The looking at point was set from 5° to 40°of 2θ, the current was 30mA, and the voltage was 35kV.

Determination of loading efficiency

Drug loading during readiness and ensuing delivery after organization are two significant properties that must be incorporated into a medication conveyance framework during their improvement as they to a great

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extent decide the proficiency of such frameworks. Drug loading is the cycle of joining of the medication into a polymer network or capsule. drug release is the opposite cycle by which the medication particles are freed from the strong stage and become accessible for retention and pharmacological activity. A 100mL volume of curcumin stacked SWCNTs were weakened with 900mL of methanol, sonicated for 1h, and thusly centrifuged at 10000rpm for 10min to take outthe SWCNTs in the determining. The supernatant game plan was utilized to pick the curcumin focus utilizing an RF5301pc spectrophoto meter (Shimadzu, Kyoto, Japan) with a repeat at 543nm.

In vitro release experiment

In a 100ml of phosphate buffer saline solution (pH 7.4) 5mg freeze dried powder test was dispersed and individual time intervals, each sample was centrifuged at 12000 rpm for 10 min and supernatant was gathered, then curcumin was extracted in desired methanol solution and after that filter this solution through 100nm pore size membrane and taken a 2ml of filtrate of SWCNTs-Cur, after that a standard curve was plot for curcumin as its function concentration and determine the release through spectrofluorometer then calculate the cumulative release rate of curcumin.

In-vitro cytotoxicity assessment

Cell cytotoxicity was assessed by tetrazolium shading-based MTT test following a once in the past declared framework [26]. MDA-MB-231, cells were kept up in RPMI-1640 medium improved with 10% warmth inactivated fetal cow-like serum and against disease specialists at 37ºC in a humidified incubation center containing 5% CO2. The cells were treated with drug stacked nanoparticles in various centers 10, 20, 30, and 40 μg/ml) for 24 hrs. Control was taken with no medication treatment. In this way, MTT was added and plates were then brought forth for another 3 hrs, the media was pipetted off and 250 IL DMSO was added. The absorbance of individual wellswas noted at 570 nm through an ELISA plate peruser at 25 ºC. Typical characteristics from three-overlap were deducted from ordinary assessment of control, what's more, the perseverance bit of cells was dictated by the equation.

Hemolytic toxicity study

The whole human blood (WHB) was taken from the endorsed pathology researchoffice in social occasion vials having hostile to coagulant property. The blood test was presented to centrifugation, by then procured red blood cells (RBCs) were stressed out and weakened 1 ml of RBCs with the extension 5 ml of refined water which was utilized as 100% hemolytic norm and the readied 100% hemolytic game plans were taken as clear for spectrophotometric appraisal and segregated into three test tubes all comprising standard hematocrit arrangement after that plain arrangement of curcumin was added on the main cylinder, SWCNTs was Hematological into the ensuing test tubes and in third chambers, CNT system was added and treated with a suspension of erythrocytes. Further, the arrangement was traversed centrifugation at 5000 rpm for 10 min and the absorbance of supernatants was discovered at 540 nm, which was utilized as a 100% hemolytic standard for recognizing the degree of hemolysis.

RESULT AND DISCUSSION

Unadulterated SWCNTs are insoluble in water, to improve their dispersibility andwater dissolvability it is treated with concentrated acids (sulphuric corrosive and nitric corrosive) so it will open its carboxylic moiety and afterward followed by the encapsulation of curcumin by ADH to make them naturally compatible. The distinguishing proof of the carboxylated CNTs have characteristic peaks of C=O extending at 1650 cm−1. The pure CNTs shows C–H extending absorption bands at 2848.9 cm−1. The peaks acquired at 3350 cm−1 recommended the presence of NH moiety in the structure. In general translation may proclaim the formation design through ADH.

Zeta potential and Particle Size assessment

Zeta potential investigations show the electric potential and variety in surface charges and stability of the colloidal system after modified formulation and the carboxylated SWCNTs (subsequent to presenting carboxylated) were shows a negative zeta potential (−14.9 mV), whereas carboxylate SWCNTs conjugated with Curcumin (CNT), the zeta potential values was found −19.5 mV. The molecule size of arranged CNT was additionally be checked and the size was discovered to be 178.21 nm, which recommended that the optimized CNT systemwas in nano level range. The CNT system was optimized at different concentrations of CNT and curcumin, which reasoned that the size of nano system was expanded with improving the concentration of CNT and the drug curcumin and the % entrapment efficiency (EE) was intrupted because of this phenomenon. The CNT was found in 178.21 nm to 298.36 nm size range, having the drug loading proficiency was decreased from 93.46% to 71.32% (Table 1).

Table 1 Optimization parameters for formulation of CNT.

S. No.	Concentration of ingredients		Particle size (nm)	% drugloading
	SWCNTs (mg)	Curcumin (mg)		

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1	10 mg	10 mg	157.65 ± 0.87 nm	89.30 ± 0.98 %
2	10 mg	20 mg	165.81 ± 0.92 nm	87.51 ± 1.10 %
3	20 mg	10 mg	172.47 ± 1.10 nm	90.37 ± 1.05 %
4	20 mg	20 mg	178.21 ± 1.15 nm	93.46 ± 1.12 %
5	30 mg	10 mg	262.54 ± 1.50 nm	83.21 ± 1.50 %
6	30 mg	20 mg	298.36 ± 1.75 nm	71.32 ± 1.71 %

Morphological assessment

For the morphological assessment of formulation TEM study was carried out and TEM pictures for functionalized SWCNTs shows small aggregates bundle (<500 nm) and the CNT formulation shows the uniform distribution and found in separated pattern. This will improve the dispersibility of formulation (Figure 1).

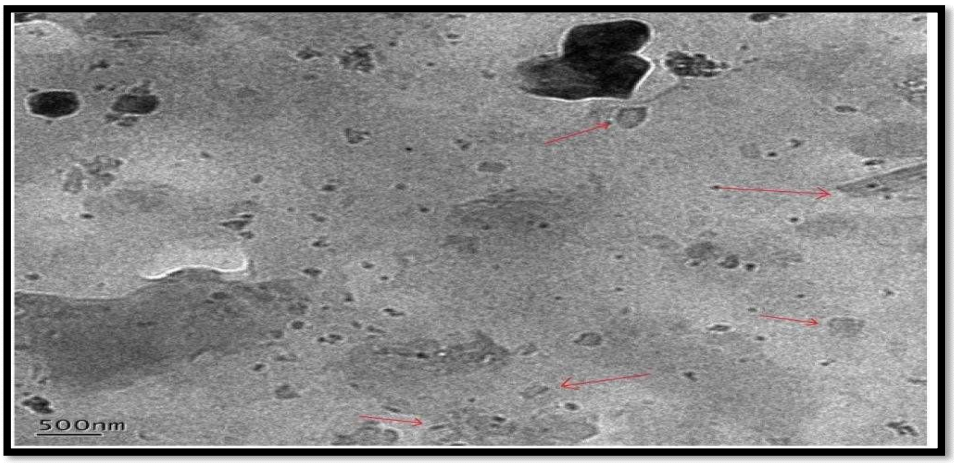

Figure 1. TEM image of CNT conjugate.

X-Ray diffraction analysis

The XRD patterns of shows that native curcumin peaks at high energy diffractionpeaks 2θ estimations of 6.6°, 8.96°, 13.83°, 16.62°, 18.29°, 35.30° and SWCNTs shows that diffraction peaks at 2θ estimations of 9.32°, 17.74°, 18.51°, 22.94°, 31.94° and 46.04°. In the XRD patterns of CUR loaded SWCNTs there were thecharacteristics curcumin peaks were noticed 6.8°, 9.16°, 12.65°, 16.60°, 17.91° and 34.20° and peaks of SWCNTs was found at 9.30°, 17.51°, 19.01°, 22.91°, 32.04° and 44.85°. The overall data suggest that, the diffractogram of CNT have the two peaks acquired from CUR and SWCNTs diffractogram. The interaction of ADH-SWCNTs with curcumin through π-π stacking association (ingested with sidewalls of ADH-SWCNTs).

Drug release and Loading efficiency of curcumin

The drug release study was completed to distinguish the delivery qualities of curcumin from CNT nano-formulation. As per the information acquired from the release study, the release of curcumin from CNT was found to be 58.36% after 120h period of time at 7.4 pH. The release rate of curcumin from CNT was found 62.35% and 69.58% if there should be an occurrence of 5.5 pH and 4.5 pH after 120 h. The release rate was higher in acidic pH then fundamental in light of breaking propensity of covalent connection between CNT was higher in acidic pH then essential pH 7.4. The loading efficiency estimated by the concentration of CURin the CUR stacked SWCNTs of 93.46±1.15%, so the loading efficiency predicted tobe as high as 90.0%. To start with, curcumin in methanol goes in atomic state withfull display with full exhibit with the surface of SWCNTs. SWCNTs have large surface areas and ADH likewise have a major structure the molecular structure of curcumin, which contains two benzene rings and a formed ethylenic linkage, the ultrasonic energy and theultrasonic probe accepted to the drug to easily adsorb ontothe SWCNTs or attach to the SWCNTs through – complexing with the benzene ring and the formation of hydrogen bonds between the carboxyl groups of the functionalized SWCNTs and the phenolic hydroxyl group of curcumin. Furthermore, the van der Waals forces among curcumin and the SWCNTs upgraded the loading of curcumin because that both curcumin and the SWCNTs are hydrophobic. During the preparation of the conjugates, we saw that the frequency, force and time of the ultrasonication applied an incredible effect on the drug loading efficiency, which may approve the proposed system.

***In-vitro* cytotoxicity assessment**

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JCHR (2023) 13(4s), 329-335 | ISSN:2251-6727

Cell viability examines dependent on MTT assay were done to find out the cytotoxic effects of CNT. For this the examination was performed with various concentrationon human breast cancer cellline MDA-MB-231. The outcome acquired from the investigation recommends that the cell development was inhibited with increasing the concentration of CNT. As indicated by the information it was concluded that the CNT have intensity to diminish the development of cancerous cell and furthermore be inhibit the cell viability at all the concentration range (Figure 2). The fundamental mechanism for the specific method of action can be attributed to enhanced drug release under acidic conditions, as per the in vitro release studies. Subsequently, this changed CNT can be considered as appropriate candidates for anticancer drug delivery.

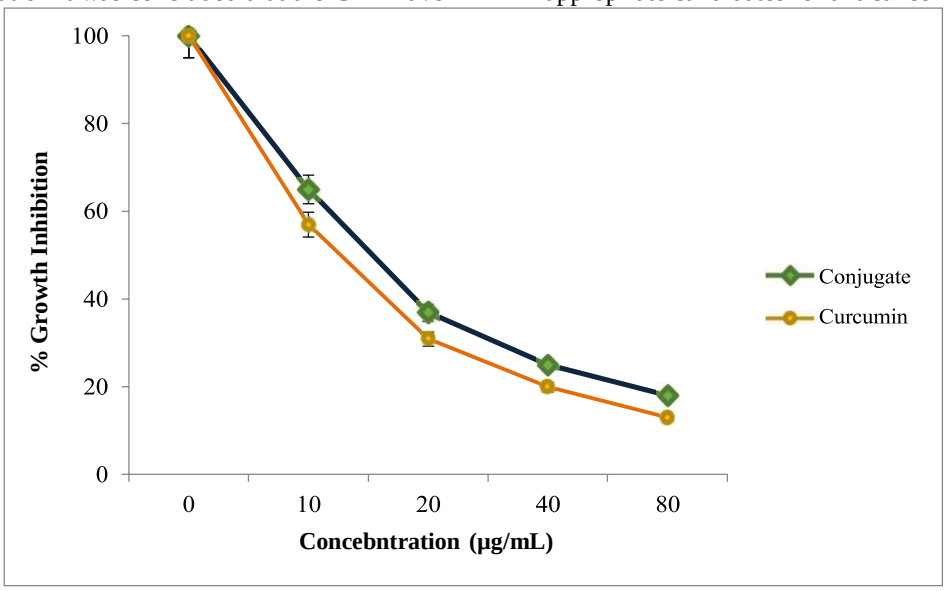

Figure 2. In-vitro cell cytotoxicity profiling of CNT against MDA-MB-231cellline.

Hemolytic toxicity study

A hemolytic toxicity study helps to determine the hemotoxic effect of the CNT.The plain curcumin, SWCNTs and CNT were found out to display hemolytic toxicity up to 38.1±0.15%, 31.1±1.1% and 7.5±0.5% individually. Probably, curcumin molecules encapsulation in the SWCNTs and the resultant deferred release causes a significant reduction in the hemolytic toxicity analysis. Moreover, CNT system have shown lower haemotoxicity in comparison with plain curcumin solution and SWCNTs solution. This may be attributed haemocompatible nature of system.

Overall finding obtained from the above analysis it was suggested that the CNT system was found in nanometric size range and having higher entrapment efficiency. The drug release characteristics also suggested that the release of curcumin from system was found in sustained release manner. The in-vitro anticancer assay suggested the anticancer potential of the CNT system and the data obtained from MTT assay, it was revealed that the system may effectively increase the population of nacrotic cells in comparison with control and should be a potential agent for cancer treatment. These findings suggest that CNT may be a promising curcumin delivery system in cancer therapies.

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JCHR (2023) 13(4s), 329-335 | ISSN:2251-6727

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