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Fabrication and anticancer evaluation of colchicine bound liposomes in colon cancer cell line

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Abstract

Treatment of cancer using nanotechnology is an interdisciplinary area of research in science. We intend to use a tumor targeting technique for colchicine-loaded liposomes. Thereafter the attachment of the drug and liposome was studied by transmission electron microscope (TEM) and the drug was successfully encapsulated into the nanoparticles. The size was (318.1 ± 4.44) nm which determined by dynamic light scattering (DLS). In vitro drug release in phosphate buffer saline (0.1M, pH 7.4) showed sustained drug release. Entrapment efficiency of colchicine-loaded liposomes was $97 \pm 0.028\%$. Colchicine-loaded liposomes had an IC50 value of 16.05 g/ml in cytotoxic study utilizing Caco-2 (colon cancer cell line) treated cells, whereas free colchicine had an IC50 value of 25.30 g/ml. When liposomes were utilized in place of free colchicine, the anticancer activity of the drug was increased.

Keywords: Liposomes; Colchicine; Nanoparticles; Colon Cancer; Drug delivery system

1. Introduction

Cancer is a disease in which some of the body's cells grow uncontrollably, rapidly and spread to other parts of the body. In normal cell cycle, human cells multiplying and grow to produce new cells as the body needs them. The old cells become damaged and new cells take their place. Sometimes this orderly process breaks down, and abnormal or damaged cells multiplying when they shouldn't. These cells may form tumors. [1-2]

Nanotechnology has a great chance to affect the treatment of cancer and many other diseases. One of the advantages of nanotechnology for cancer treatment is tumor targeting using different formulation techniques. [1]. Regarding to cancer several studies showed that nano particles have been successfully used for the treatments. In this regard, nanocarriers have facilitated the targeted delivery of chemotherapeutics as the liposomal formulations of anticancer drugs have the ability to improve the pharmacokinetics and pharmacodynamics of their loaded drugs resulting in the efficient inhibition of malignant tumors. [3]

Liposomes are one of the nanoparticles which made of phospholipid as cell membrane and have the ability to encapsulate hydrophilic and hydrophobic drugs. It can be formed with different sizes with many techniques. [4] One of these techniques is ethanol injection which produce single bilayer liposome by injection of a small amount of ethanolic phase into the aqueous phase under magnetic stirrer until the ethanol evaporate. [5]

Colchicine has been used for treatment of gout attacks but recently we found that it can still be used as a compound of anticancer drugs. [6-7] Colchicine can limit mitochondrial metabolism in cancer cells by increase cellular free tubulin. [8] Because of the toxicity of colchicine, the clinical applications have been limited. In comparison of free drug, the nano formula enhances the therapeutic efficiency of colchicine. [9]

Few data have been published regarding the interaction of Colchicine with phospholipids. To date, the anti-tumor activity of Colchicine in free and nano liposomal forms towards Caco-2 (colon cancer cell line) has not been much studied.

The aim of the present study is to investigate how Colchicine modulate the physical structural properties of model lipid membranes and to estimate the subtle perturbation of the lipid bilayer structure using transmission electron microscopy (TEM), Zeta potential, as well as Dynamic light scattering (DLS). The work also evaluates the cytotoxic efficacy of Colchicine with liposomes against human colon carcinoma cell line (Caco-2) (*in-vitro* study).

2. Experimental

2.1 Materials

Colchicine (Sigma Aldrich, Germany), absolute ethanol (Merck, Germany), phospholipon 90G (lipoid, Ludwigshafen, Germany) and Millipore water.

2.2 Liposomal preparation using ethanol injection technique

Liposomal nanoparticles were prepared using a ethanol injection method. Colchicine was first solubilized in a water miscible organic solvent (Ethanol) with concentration 0.1% w/v then 10% w/v of phospholipoid 90 G was added to the ethanol solution, the mixture was heated at 37 °C while being stirred on Digital plate magnetic stirrer, (DAIHAN Scientific, Korea) for 10 min (at 500 rpm) until complete solubilization of the drug. The mixture was injected into aqueous phase while the latter was maintained at room temperature on a magnetic stirrer (DAIHAN Scientific, Korea) at 1000 rpm. Subsequently, the suspension was left on the stirrer for 30 min till the organic solvent was evaporated then collected, and physical characterization was performed.

2.3 Transmission electron microscope

High Resolution Transmission Electron Microscope (HRTEM): (JEM-2100) with selected area electron diffraction capability (SAED) was used to view the prepared colchicine-loaded liposome in colloidal form. A drop of the nanoparticles was placed on glass slide and dried at room temperature then pictured by a fixed camera in the TEM device (JEM-2100).

2.4 Particle size characterization

The prepared particles were analyzed for their particle size and size distribution in terms of the average volume diameters and polydispersity index by photon correlation spectroscopy using particle size analyzer Dynamic Light Scattering (DLS) (Zetasizer Nano ZN, Malvern Panalytical Ltd, United Kingdom)

at fixed angle of 173° at 25° C. Samples were analyzed freshly in triplicate after suitable dilution using deionized water. The same equipment was used for the determination of zeta potential.

2.5 Invitro drug release

1 ml of the medicated formula (liposomal Colchicine) was added to a dialysis bag (12-14KD cut off, Sigma Aldrich, Germany).[10] The experiment was done triplicate. The dialysis bag was sealed properly both from top and bottom and inserted into 25 ml of 0.1 M phosphate buffer saline with pH 7.4 for 24 h in proper jar . The whole system was fixed in a shaking incubator (Jeio tech SI-300, SEOUL, KOREA) rotating at 100 rpm with temperature adjusted to 37 °C. Samples were withdrawn at 0.25, 0.5, 1, 2, 4, 6, 8 and 24 hr, 1 ml medium sample was withdrawn and immediately replaced with another 1 ml of equally warmed buffer for 24 h. [10]

2.6 Entrapment efficiency

A freshly prepared nanoparticles were added to centrifugal filter unit (Amicron Ultra -10 K, Merk Millipore, Tullagreen, Carrigtwohill) and centrifuged at 10000 rpm for 30 min at 4 °C using cooling centrifuge, (NEYA16R, India) then supernatant was collected to separate the non-entrapped drug. [11] The EE% of colchicine was determined by indirect method using HPLC (Waters 2690 alliance hplc system, photodiode array detector). using the following chromatographic conditions; with the column temperature adjusted at 25 °C. The mobile phase consisted of 70:30 0.1% OPA: pure acetonitrile, with a flow rate of 1 ml/ min. Inertsil column (150mm, 4.6mm, 5μm) was used for the analysis and peak areas were determined at a retention time of 5.5 min (EE% was calculated according to the following equation: EE% = (Entrapped drug concentration / Total drug concentration) × 100 The drug loading was calculated. [12]

2.7 MTT cytotoxicity assay

Cytotoxicity of the free, the entrapped colchicine and pure colchicine were determined by measuring the inhibition of cell growth in caco-2 cancer cell lines by MTT cytotoxicity assay. To assess the half-maximal cytotoxic concentration (IC50), stock solutions of the tested compound were prepared in media DMEM (Merck, Germany). The cytotoxic activity of the sample was tested in CaCo2 (colorectal cancer) cell line by using the 3-(4, 5-dimethylthiazol - 2-yl)-2, 5-diphenyltetrazolium bromide (MTT) method with minor modifications. Briefly, the cells were seeded in 96-well plates (100 μl/well at a density of 3×105 cells/ml) and incubated for 24 h at 37 °C in 5% CO2. After 24 h, cells were treated with various

concentrations of the tested compounds in triplicates. After 48 h, the supernatant was discarded, and cell monolayers were washed with sterile 1x phosphate buffer saline (PBS) 3 times, and MTT solution (20 µl of 5 mg/ml stock solution) was added to each well and incubated at 37 °C for 4 h followed by medium aspiration. In each well, the formed formazan crystals were dissolved with 200 µl of acidified isopropanol (0.04 M HCl in absolute isopropanol = 0.073 ml HCL in 50 ml isopropanol). The absorbance of formazan solutions was measured at λmax 540 nm with 620 nm as a reference wavelength using a multi-well plate reader (BMGLABTECH®FLUOstar Omega, Germany). The percentage of cytotoxicity compared to the untreated cells was determined with the following equation .

The plot of % cytotoxicity versus sample concentration was used to calculate the concentration which exhibited 50% cytotoxicity (TC50). [13-14]

$$\% \, Cytotoxicity = \frac{(absorbance\ of\ cells\ without\ treatment - absorbance\ of\ cells\ with\ treatment)}{(absorbance\ of\ cells\ without\ treatment)} X100$$

3. Result and discussion

The nano formula was found translucent and it was clear after dilution with deionized water, the system was in nanosized range of (**318.1 ± 4.44**) nm. The entrapment efficiency percentage was found to be **97 ± 0.028 %** for colchicine-loaded liposomes.

The morphology of every liposome generated in this work was practically spherical in shape, well diffused, and less aggregated for enclosed vesicles, according to TEM images, as shown in (**Figure 1**). Colchicine can physically be linked to disturbing the liposomes' surface membrane packing property, according to TEM results. Colchicine increased the distance between adjacent bilayers in liposomes (**Figure 1**), resulting in larger liposomes. Stronger colchicine interactions with the lipid bilayer of liposomes through hydrogen bonding may be the cause of the increase in particle size. It has been suggested that colchicine might be placed in the bilayer's hydrophobic area.

A typical size distribution graph for both empty and colchicine-encapsulated liposomes is shown in Figure 2. According to (**Figure 2A**), the mean size diameter of the empty liposomal sample was 225.6 ± 204.4 nm, with a PDI of 0.453. Colchicine was added to liposomes, increasing their calculated mean size diameter from 225.6 ± 204.4 nm to 313 ± 219.5 nm with 0.479 PDI, as shown in (**Figure 2B**). The results of DLS measurements (**Figure 2**) are consistent with the particle size depicted by TEM in (**Figure 1**). These findings suggest that colchicine molecules may be physically interacted with liposomes at the surface and tend to be buried in lipid bilayers, which may help to explain why the size is increased.

The magnitude of the zeta potential determines the potential stability of the colloidal system. A more stable colloidal dispersion results from an increase in particle repulsion as the zeta potential rises. If every particle in a suspension has a significant negative or positive zeta potential, they will all reject one another and have no desire to unite.[15]

Empty liposomes showed negative zeta potential (-**46.7 ± 5.57 mV**), in agreement with the observation of other.[16,17,18,19] Because colchicine was incorporated into the liposomal membranes, colchicine-loaded liposomes had a lower negatively zeta potential (**-44.6 ± 6.61 mV**) than empty liposomes (**Figure 3**). Particles with zeta potentials greater than or equal to +30 mV or greater than or equal to -30 mV are generally regarded stable.

For the in-vitro release of Colchicine from the phospholipoid liposomes, various estimates of drug delivery performance were made. By incubating in PBS at 37° C versus time in hours, the leakage of Colchicine from liposomes was examined. Further examination of (**Figure 4**) demonstrates that, in the case of Colchicine, the first stage of continuous drug release is visible within the first two hours. Colchicine's formulation is comparatively stable at 37°C, releasing roughly 18–38% over the first two hours, then at a relatively modest rate over the following hours (39%). Because liposome-doped colchicine exhibits improved in-vitro release stability (approximately 39% between the third and 24th hours), there is less colchicine leakage, which would result in higher colchicine retention. Colchicine vesicles exhibit noticeably better in-vitro release stability (P< 0.01) as well as a decreased leakage rate that is accompanied by greater drug retention.
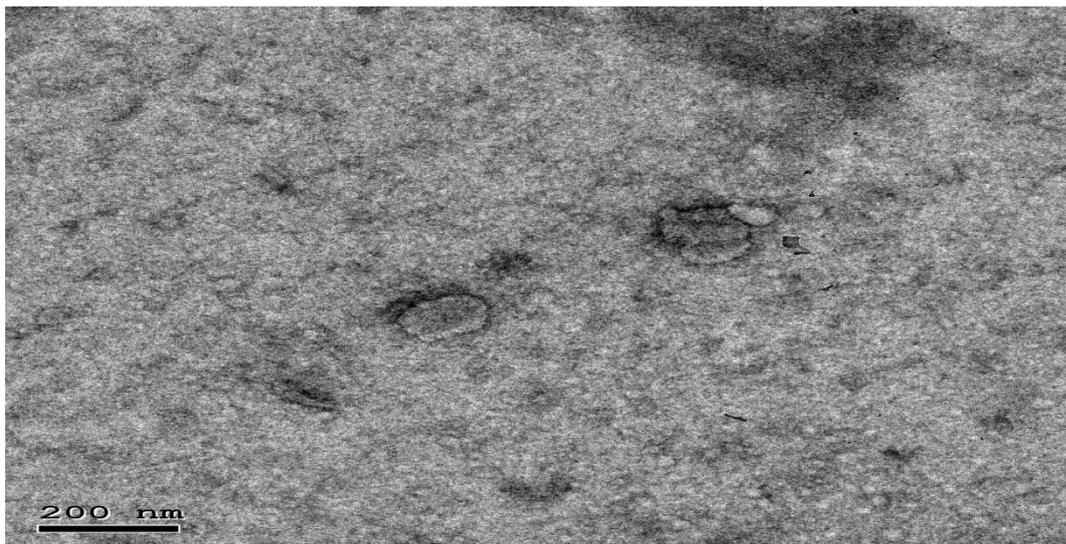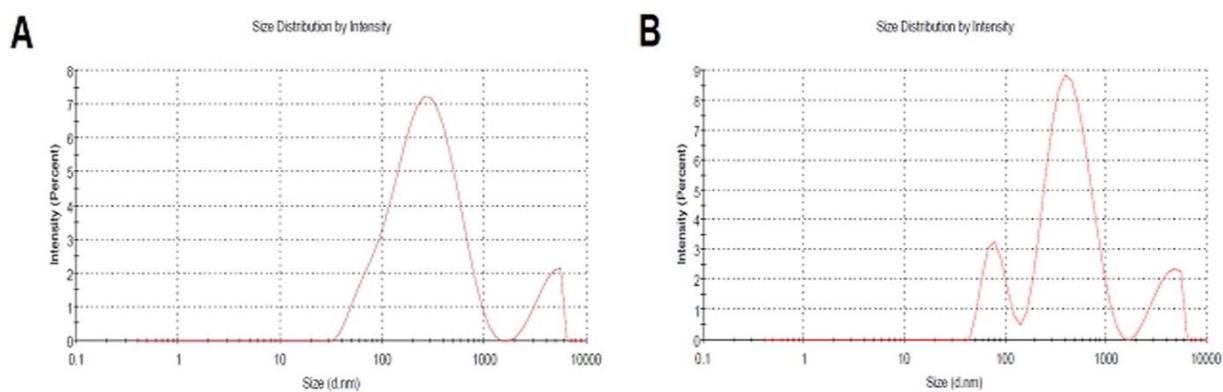
Figure 1: TEM image for Colchicine-loaded liposomes

Figure 2: Liposomes size distribution measured by dynamic light scattering (DLS) for (A) empty Liposomes (B) colchicine-loaded liposomes.
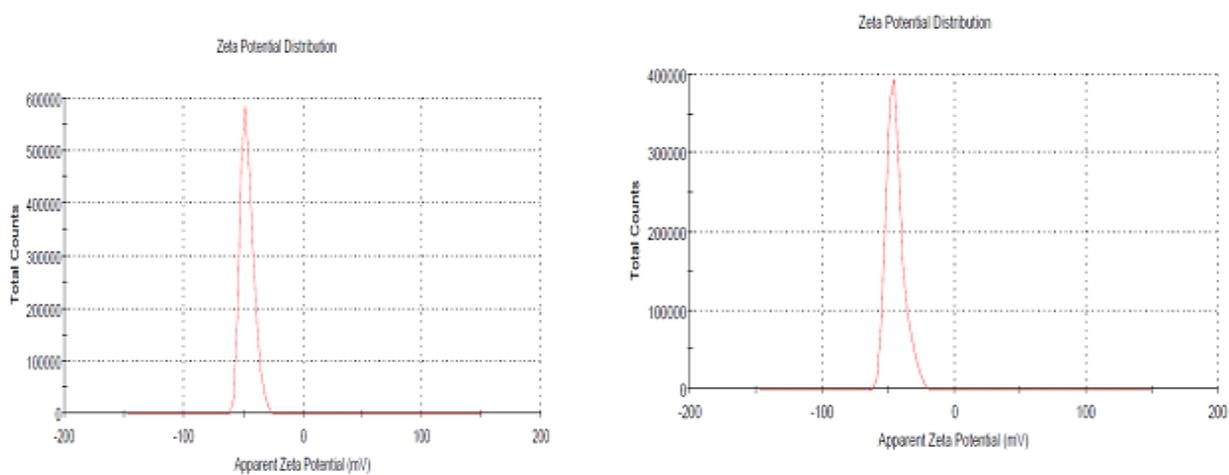

Figure 3: Zeta potential measurements for (A) empty liposomes (B) colchicine-loaded liposomes.

Utilizing a cell viability (In Vitro MTT cytotoxicity assay) test against colon cancer (CaCo2) cell lines at various drug concentrations of Colchicine-loaded liposomes and empty liposomes, the effectiveness of the drug delivery method was investigated. Cells that had not been treated served as controls with no drugs present at all. At 48 hours, the test was finished, and cell viability measures were taken. The same series of various drug doses, ranging from 0-500 g/ml, were independently incubated with the CaCo2 cancer cell lines for 48 hours, as shown in **Figure 5**.

According to cell viability experiments, compared to free Colchicine, empty liposomes or liposomes filled with the drug exhibited the highest level of cytotoxicity against the CaCo2 cell line. 48 hours after incubation, CaCo2 treated cells had a cell viability of about 3.93% at the highest Colchicine-doped liposomes concentration (500 g/ml). When cells were given empty liposome treatment, their vitality stayed at about 2.218 percent. Free Colchicine has an approximate 18.726% cell viability at the same concentration (500 g/ml).

The significant drop in cell viability in Colchicine-loaded liposomes treated cells as compared to free Colchicine may be explained by the prolonged release of Colchicine from liposomes. As a result, the amount of released drug increases together with the concentration of the encapsulated drug, which lowers cell viability.

It's interesting to note that empty liposomes treated with the same concentration (500 g/ml) showed a significant reduction in cell viability against the CaCo2 cell line. The viability of the cells was around 2%. This demonstrates that the tested cells were damaged by the liposomes' high concentration. The interferences with cell proliferation that we discovered in association with several phosphatidylcholines may be caused by many methods since there is no clear-cut rule regarding toxicity. Further research will concentrate on a more thorough analysis of these fundamental mechanisms, which are currently the subject of conjecture.

Cytotoxic activity among various drug formulations at higher concentrations displayed an order of empty liposomes > Colchicine -doped liposomes > free Colchicine according to (**Figure 5).**

Colchicine-doped liposomes showed cell viability 16.67% at the lower concentration of around 100 µg/ml when compared to its free form, which was about 40.5%, and 24.87% when empty liposomes were used, as shown in (**Figure 5)**.
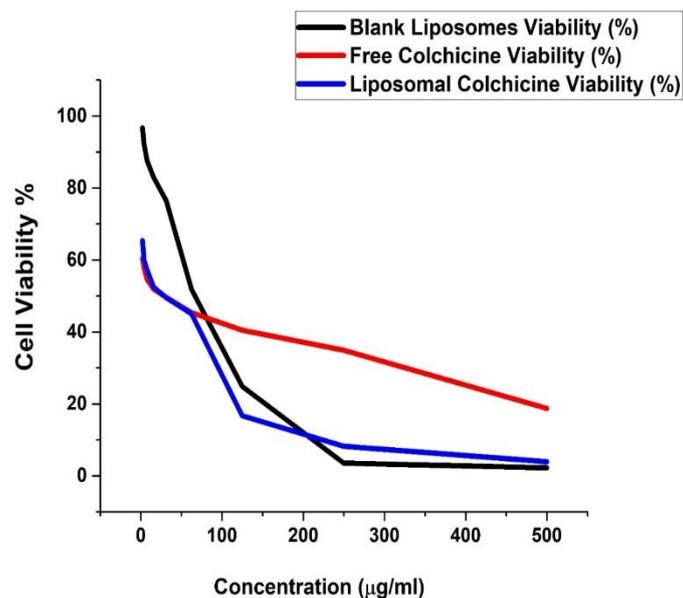

Figure 4: Cytotoxicity of free Colchicine, Colchicine-loaded liposomes and empty liposomes against colon carcinoma (CaCo2) cell line; incubated for 48 h with different concentrations (starting from 0-500 µg/ml). The cell viability was determined using the MTT assay. The data represent mean ± standard error of triplicate experiments.

In a cytotoxic study using CaCo2-treated cells, the IC50 value for Colchicine-doped liposomes was 16.05 µg/ml, but the value for CaCo2-treated cells with free Colchicine was 25.30 µg/ml. It is important to note that the efficacy of Colchicine was increased by around two times when liposomes were used instead of unloaded Colchicine. Liposome-encapsulated chemotherapy drugs work more actively and with less effort to provide the intended effects. A 2-fold boost in anticancer activity was discovered through additional investigation into the possibilities of improving the effects of colchicine by using liposomes as nanoscale carriers.

This increased efficacy may be attributed to the drug's lipo-solubilized form, which results from its entrapment within many lipoidal domains of vesicles. Additionally, the cell membrane and the phospholipid lamella in the vesicular periphery may combine, allowing for the uptake of vesicular content.

IC_{50} value for CaCo2 treated cells with empty liposomes was 59.46 µg/ml. Based on above results, the encapsulation of Colchicine into liposomes showed the highest therapeutic efficacy against CaCo2 cell line, depending on the cancer cells type, **Figure 6**.
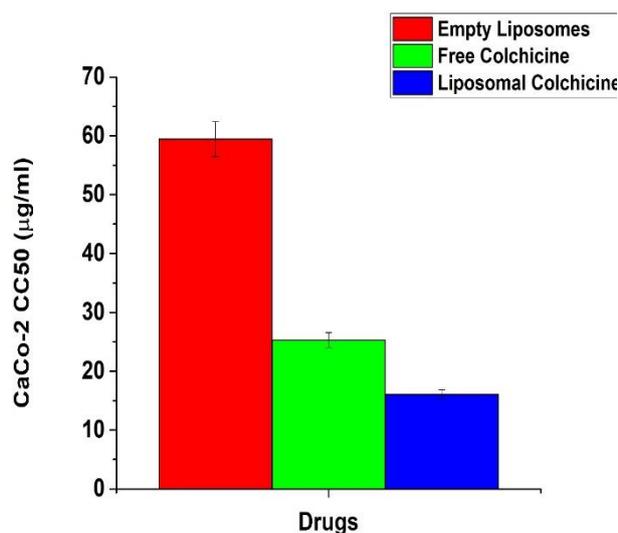
Figure 5: IC₅₀ chart with significant values for free Colchicine, Colchicine-loaded liposomes and empty liposomes against colon carcinoma (CaCo2) cell line by using MTT assay, 48 h post-treatment.

Conclusion

The present study focused on the use of colchicine as an anticancer drug. In this study, an improved liposomes preparation method has been proposed for colchicine with high encapsulation capacity to enhance anticancer activity against the CaCo2 cancer cell line. Our study's findings suggest that colchicine-loaded liposomes might improve colchicine's therapeutic efficacy. These results may help in the development and design of Colchicine-loaded liposomes for the treatment of colon cancer. Future research should explore the different ways that liposomes and other cancer medicines can be combined.

Conflict of interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this paper.

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