



SCREENING ANTI-CANCER ACTIVITY OF COLCHICINE LOADED CHITOSAN NANOPARTICLES

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ABSTRACT

Introduction: In the present study we reported quick and easy method for the synthesis of chitosan nanoparticles (CNPs) and Colchicine loaded chitosan nanoparticles (CCNPs) by using ionic gelation method. **Materials and Methods:** Synthesized nanoparticles were characterized by FTIR, SEM, XRD and Zeta Potential. **Results:** FTIR analysis showed presence of various functional groups. Nanoparticle size (200nm) and stability (+10mV) of CCNPs was reported from the results of SEM and Zeta Potential respectively. Entrapment efficiency, Loading efficiency and percentage yield was found to be 60.7%, 48.8% and 20.7% respectively. Debye-Scherrer equation showed particle size as 34.65 nm calculated from XRD spectrum. Anticancer activity was screened by MTT assay on HeLa, SKOV3, PC3, Panc-1 and MDAMB231. IC-50 value was found to be 58.74±9.58, 64.55±9.50 for pure colchicine and CCNPs against HeLa cell line respectively and 89.06±25.58, 92.25±15.58 against SKOV3 cell lines respectively. **Conclusion:** CCNPs were found to be more potent than pure colchicine. Thus our study discovered CCNPs as a potent source of nanomedicine for the treatment of various Cancers.

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Introduction

Cancer is a group of diseases characterized by unregulated growth of cells, invasion and spread of cells from site of origin or primary site, to other sites in the body. Cancer cells have the ability to create their own blood supply, break away from the organ of origin, travel, and spread to other organs of the body [1]. Cancer is the second leading cause of death globally, and is responsible for an estimated 9.6 million deaths in 2018. In present scenario, a common method used for the treatment of cancer is chemotherapy, but it is often associated with large number of drawbacks such as, selection of anti-cancer drugs, multidrug resistance, high drug toxicity, and undesirable side effects to normal tissue [2, 3]. To this regard, to overcome the drug resistance, side effect, toxicity and increase the therapeutic index is loading of anticancer drugs in nanoparticles, which are a new and innovative technology that overcome a lot of problems related to drug delivery system, retention and prevention through barrier.

Presently, different metallic nanomaterials are being produced using copper, zinc, titanium, magnesium, gold, alginate and silver. Nanoparticles are being used for diverse purposes, from medical treatments, using various branches of industry production such as solar and oxide fuel batteries for energy storage, to wide incorporation into diverse materials of everyday use [4].

Chitosan[β-(1-4)-2-amino-2-deoxy-D-glucose] is a polymer obtained by the de-acetylation of chitin which is a fibrous substance present in the exoskeleton of arthropods such as crustaceans like crabs, lobsters and shrimps, scales of fish and cell walls of fungi. It is a naturally occurring linear biodegradable polymer that is made up of N-acetyl-D-glucosamine and D-glucosamine [5]. CNPs prepared by electrostatic interaction between the cationic chitosan and anionic counter ions STPP have an ideal as one of the most promising carrier system for drug delivery [6]. Chitosan has been used widely in pharmaceutical industry in drug delivery systems in various forms, like tablets, microspheres, micelles, vaccines, nucleic acids, hydrogels, conjugates and nanoparticles. Chitosan and its derivatives can be used in drug delivery systems in both implantable as well as injectable forms [7].

Colchicine is lipid-soluble tricyclic alkaloid extracted from plant genus *Colchicum Autumnale* and has a half-life of 20 to 40 hours and bioavailability ranging from 24% to 88%. Colchicine has a melting point of 150-160 °C. Colchicine will reach

higher concentrations within leukocytes than in plasma, and persists there for several days after ingestion [8, 9]. Colchicine has many therapeutic uses like treatment of Rheumatic diseases such as Gout, Osteoarthritis and Familial Mediterranean fever and also used in treatment of Non-rheumatic diseases like pericarditis, atherosclerosis, cardiovascular diseases and also hepatic diseases [10- 13].

In present study, our attention has given for potential delivery systems to increase the drugs bioavailability by enhancing the residential time which subsequently facilitate the absorption of drug through adhesion with the cellular surface. In this view, the best considered strategy to achieve enhanced bioavailability of colchicine is to load it within CNP's. Thus, chitosan based nanoparticles delivery system can solubilize colchicine in aqueous environment and protect it from hydrolytic degradation and delivers colchicine in a controlled manner. In this way, it will improve the bioavailability of delivered colchicine for maximizing tumor therapeutic efficacy and reduce the pharmacokinetic problems.

Materials and Methods

Materials

Chitosan (Chemsworth, India) was used as a polymer and Colchicine (Essel Laboratories, India) was used as major drug and STPP as gelling agent (Qualikems fine chemical limited, India). Ethanol was used as suspending agent, and acetic acid (Pallav Chemicals and solvents Pvt. Ltd, India) was used as solvent. The water used in all experiments was double distilled and laboratory grade.

Preparation of CNPs and CCNPs

In brief, an aqueous solution of 9 ml of STPP (1mg/ml) was added dropwise to 17.5 ml of chitosan solution (1 mg/ml) with and without colchicine (4 mg/ml). The chitosan solution was prepared by dissolving in a 1% acetic acid and the pH was adjusted to 5.0 with 0.1 M NaOH. It was observed that the formation of nanoparticles without any aggregation, even after the particles were left overnight on magnetic stirrer. After overnight stirring the CCNPs were collected by centrifugation at 8000 rpm at 4°C for 30 min and the pellet was washed with 10% aqueous ethanol. The pellets were then resuspended in 10% aqueous ethanol and then stored at 4°C. This formulation was selected for further studies and characterization as well as for entrapment of colchicine.

Entrapment Efficiencies (EE%), Loading Efficiencies (LE%) and Percentage yield of CCNP's

A known quantity of CCNPs (3mg) was dissolved in 10% DDW and solution was sonicated at 40% amplitude for 30sec intervals up to 10 min; the resulting solution was centrifuged at 3000 rpm for 15 min at 4°C. The amount of colchicine within the supernatant was quantified spectrophotometrically at an absorption maximum of 354 nm which corresponds to peak absorption of standard colchicine and calculated by calibration curve of standard colchicine.

$$EE\% = \left(\frac{\text{Total amount of colchicine in pellet}}{\text{Initial amount of colchicine taken for loading studies}} \right) 100$$

$$LE\% = \left(\frac{\text{Total amount of colchicine trapped within the pellet}}{\text{Yield of CCNP's}} \right) 100$$

$$\% \text{ Yield} = \left(\frac{W_1}{W_2} \right) 100$$

Where W_1 = Dried Wt. of NP's, W_2 = Wt. of colchicine + Wt. of STPP + Wt. of Chitosan

Zeta potential analysis of CCNP'

Light scattering method was employed to detect stability of CCNP's particles at room temperature in suspension form using MALVERN (MAL1004428).

SEM mediated assessment of CCNP's

Morphology, shape and size of synthesized CCNP's was determined by HITACHI (3000N). For this small quantity of CCNP's was dropped on carbon coated copper grid, blotting paper was used to remove extra solution and final grid was used for SEM analysis.

FTIR mediated assessment of CCNP's

This was recorded using Fourier Transform Infrared Spectrophotometer (BRUKER ALPHA) operated in the range from 4000 to 500 cm^{-1} . For FTIR-mediated assessment obtained CCNP's are diluted in DDW, centrifuged at 5000 rpm for 15 min to remove the unbound moieties. Supernatant obtained after centrifugation is discarded, pellet is resuspended in DDW and procedure is repeated for 3 times, final pellet obtained is dried in hot air oven at 60 °C to obtain dried and pure form of CCNP's which are then used for characterization.

X- ray Diffraction Study (XRD)

XRD analysis was recorded to know the crystallographic structure of CCNP's. The structural patterns of CCNP's were measured by X-Ray Diffractometer. The measurements were performed at a voltage of 40 kV and 30 mA. The scanned 2-Theta scale (Shimadzu, XRD7000) was set from 2.0000° to 50.0014°, Step=0.0053°, step time = 13.93 with Anode Cu, wavelength- 1.5406 Crystallographic structure of CCNP's was calculated by using peaks of XRD. Crystallographic structure

of CCNP's was calculated by using peaks of XRD. Average size of CCNP's was assessed by using the Debye-Scherrer equation.

$$D = \frac{k\lambda}{\beta \cos\theta}$$

Where, D = Average size of CCNP's, k = constant (0.94), λ = x-ray wavelength (1.5406), β = Full width at half the maximum (FWHM), θ = Bragg's angle in degrees.

Anti-Proliferative activity of CCNP's by MTT assay against HeLa, PC3, SKOV3, Panc-1 and MDAMB231

All cell lines used in this study were purchased from the American Type Culture Collection (ATCC, United States). MDAMB231 (metastasis breast cancer cell line), SKOV3 (ovarian cancer cell line), Panc-1 (pancreatic cancer cell line) and HeLa (cervical cancer cell line) were grown in Dulbecco's modified Eagle's medium (containing 10% FBS in a humidified atmosphere of 5% CO₂ at 37 °C). PC3 (prostate cancer cell line) cells were cultured in medium Roswell Park Memorial Institute (RPMI) containing non-essential amino acids, 1 mM sodium pyruvate, 10 mg/mL bovine insulin, and 10% FBS. Cells were trypsinized when sub-confluent from T25 flasks/60 mm dishes and seeded in 96-wel plates. The synthesized test compounds were evaluated for their in vitro anti-proliferative activity in five different human cancer cell lines including HeLa, PC3, Panc⁻¹, MDAMB231 and SKOV3. A protocol of 48 h continuous drug exposure was used, and MTT cell proliferation assay was used to estimate cell viability or growth. The cell lines were grown in their respective media and were seeded into 96-well microtiter plates in 100 μ L aliquots at plating densities depending on the doubling time of individual cell lines. The microtiter plates were incubated at 37 °C, 5% CO₂, 95% air, and 100% relative humidity for 24 h prior to addition of experimental drugs. Each compound was treated at 50 μ g/ml and was done in triplicate wells. Plates were incubated further for 48 h, and the assay was terminated by the addition of 10 μ L of 5% MTT and incubated for 60 min at 37 °C. Later, the plates were air-dried. Bound stain was subsequently eluted with 100 μ L of DMSO, and the absorbance was read on a multimode plate reader (Perkin Elmer) at a wavelength of 560 nm.

Results and Discussion

Entrapment efficiency and Loading efficiency

The ideal synthesis of the nanoparticles was prepared with chitosan (1 mg/ml) and STPP (1 mg/ml). Standard curve of colchicine was obtained and R² value was found to be 0.999. Entrapment and Loading efficiencies are the two main parameters which are estimated by UV Visible spectrophotometer, used to determine chitosan as nano-carrier. EE% was found to be 60.7%, LE% was found to be 48.8%, % yield of CCNPs was found to be 20.7 %.

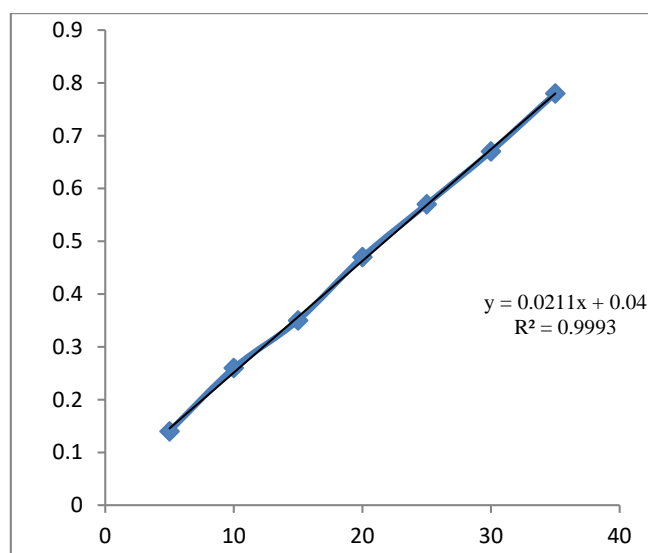


Figure 1: Standard curve of colchicine

Zeta Potential mediated assessment of CCNPs

Zeta Potential was found to be +10 mV. Positive value justifies the incipient stability and also prevents the agglomeration of synthesized CCNPs. Zeta potential graph is shown below in Fig. 2.

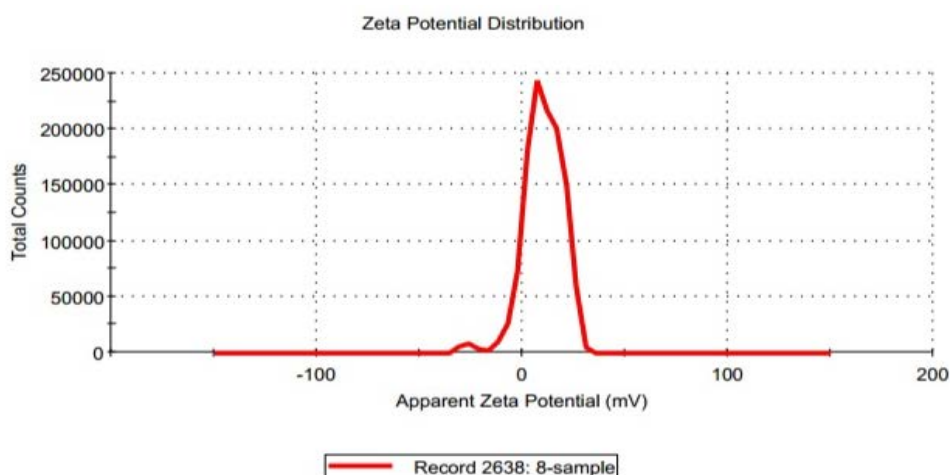


Figure 2: Zeta potential of CCNPs

SEM mediated assessment of CCNPs

Fig. 3 depicts the topography and surface morphology of CCNPs. SEM revealed the size of CCNPs as 200 nm which falls within the nano range.

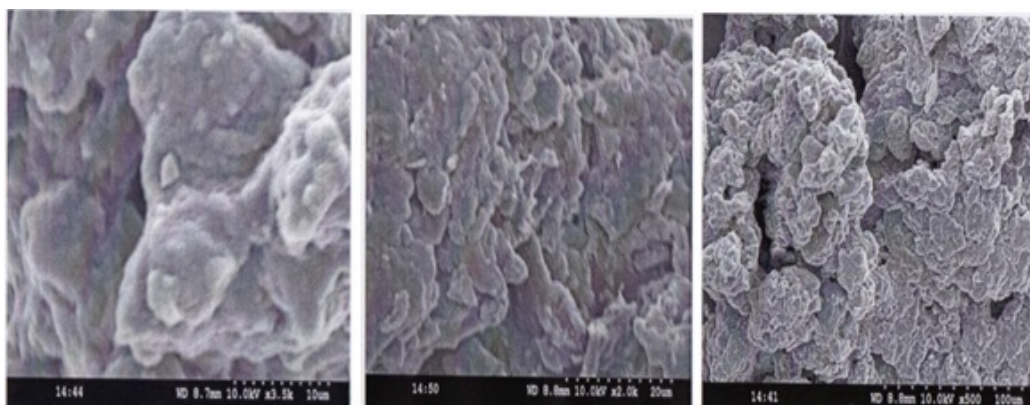


Figure 3: SEM Pictograms of CCNP's at 100µm, 20µm, 10µm

FTIR mediated assessment of CCNPs

FTIR analysis was carried out to identify various functional groups. CNP showed peaks at 3321.72, 1635.33, 1540.25, 1381.52, 1309.40, 1218.70, 1152.83, 1073.92, 1025.49 and 577.62 cm^{-1} (Fig. 4). O-H Stretching of intermolecular Hydrogen bonds showed peak at 3321.72, C-H^{*} stretching of aldehydes at 1635.33, N-H Bending of secondary amides at 1540.25, C-O stretching of tertiary alcohols at 1381.52 & primary alcohols at 1309.40 and C-C stretching at 1218.70, 1152.83, 1073.92, 1025.49 and C-Br Stretching at 577.62 cm^{-1} .

Pure chitosan has the peaks with functional groups which are similar to CNPs. They are 1381.03 C-O stretching of tertiary alcohols and 1152.83 C-C stretching of C-C bond. Pure colchicine showed peaks at 3262.97, 2939.95, 2355.62, 1508.09, 1544.81, 1488.78, 1321.96, 1194.69, 1094.4, 1019, 805, 600 and 485 which corresponds various functional groups. CCNPs showed peaks at 3278.96, 1635.50, 1539.72, 1380.73, 1312.12, 1220.53, 1152.65, 1071.51, 1023.50, 966.36 and 894.62 cm^{-1} (Fig. 5). The various functional groups are N-H Stretching of 2° amides at 3278.96, C-H^{*} stretching of aldehydes at 1635.50, N-H bending of secondary amides at 1539.72, C-H bending of $-\text{C}(\text{CH}_3)_2$ at 1380.73, N=O stretching of Ar-NO₂ at 1312.12, C-O stretching of C=C-O-C at 1220.53, C-C stretching of C-C at 1152.65, 1071.51, 1023.50 and C-H bending of disubstituted groups at 966.36, and 894.62 cm^{-1} . Comparison of both CNP & CCNPs peaks shows similar functional groups. The similar peaks are 1635.33, 1152.83, 1073.92 and 1025.49 cm^{-1} . Further comparison with Pure Colchicine CCNPs show similar functional groups at 1539.72, 1071.5 cm^{-1} .

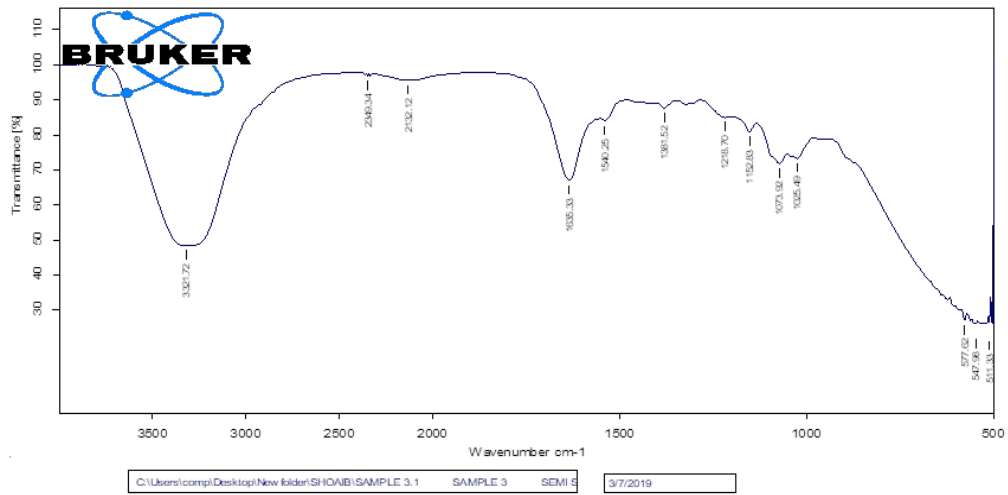


Figure 4: FTIR analysis of chitosan nanoparticles

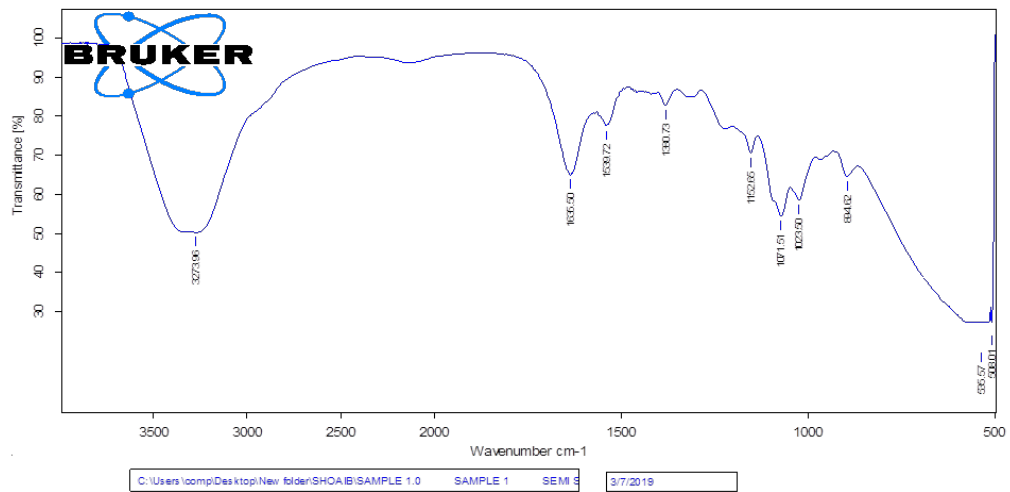


Figure 5: FTIR analysis of colchicine loaded chitosan nanoparticles

XRD studies of CCNPs

Fig. 6 depicts the XRD pattern of synthesized CCNPs. 2θ peaks are observed at 26.80 corresponds 13.4 planes of Bragg's reflection. The size of CCNPs was found to be 34.65 nm.

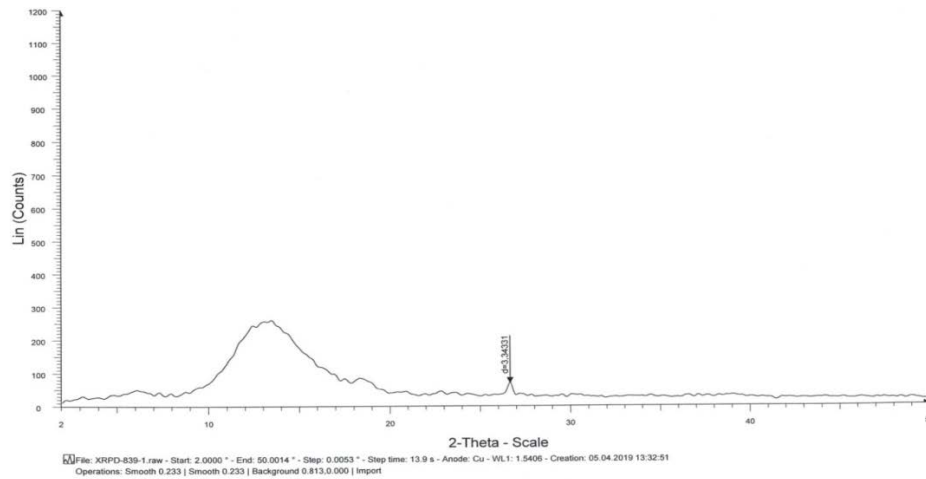


Figure 6: XRD pattern of CCNP's

Anti-Proliferative activity of CCNPs by MTT assay against HeLa, PC3, SKOV3, Panc-1 and MDAMB231

Cytotoxicity of the PC and CCNPs were evaluated and IC-50 values were found to be 58.74±9.58, and 64.55±9.5 for HeLa cell lines respectively, and 89.06±25.58, and 92.25±15.58 against SKOV3 cell lines respectively. The IC₅₀ values of different cell lines are detailed in Table 1. CCNPs are found to be more effective when compared to PC. Moreover, both PC and CCNPs are not active against MDAMB231, Panc⁻¹ and PC3.

Table 1: Anti-Proliferative activity of CCNP's by MTT assay against HeLa, PC3, SKOV3, Panc⁻¹ and MDAMB231

Materials	IC-50 values of against different Cell lines				
	HeLa	MDAMB231	Panc ⁻¹	PC3	SKOV3
PC (50µg/ml)	58.74±9.58	NA	NA	NA	89.06±25.58
CCNP's (50µg/ml)	64.55±9.5	NA	NA	NA	92.25±15.58

PC- Pure colchicine, CCNP's- Colchicine loaded chitosan nanoparticles

Conclusion

In recent years the traditional use of natural compounds especially of plant origin received much attention as they are well tested for their efficacy and generally believed to be safe for human use. CCNPs were successfully synthesized by ionic gelation method and further characterized by FTIR, SEM, XRD and Zeta Potential. Anti-cancer activity was evaluated by using MTT assay. CCNPs showed activity against HeLa and SKOV3, but it is not active against MDAMB231, Panc⁻¹ and PC3. Thus based on results we can conclude that nanoparticle formulation showed better anti-cancer potential when compared with PC. Further studies should be carried out to screen in-vivo anti-cancer activity of CCNPs.

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Conflicts of Interest

None of the sharing authors claimed any conflict of interest.

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