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# Isolongifolene-Loaded Chitosan Nanoparticles Synthesis And Characterization For Cancer Treatment

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**ABSTRACT**

The advancements of nanoparticle-based treatment approaches have revolutionized the cancer treatment strategy, as novel approaches have been adopted with success in the management of this disease in extending the time to the onset of the disease. Compared to other therapeutic leads used in conventional drug delivery nanoparticulate systems exhibit excellent penetrating power based on several factors and are most affecting in disease control. In the current study, isolongifolene - loaded Chitosan nanoparticles have been formulated, synthesized and then characterized using Fourier transform infrared spectroscopy, X -ray Diffraction, Scanning Electron Microscopy, and Transmission Electron Microscopy. In addition, the characterized chitosan nanoformulation was tested in terms of bleeding time, plasma degradation, and also followed in simulated gastrointestinal media. Chitosan nanoparticles containing isolongifolene were shown to be stable in plasma and sustained the release profile. Therefore, chitosan loaded nanoparticles could be a suitable companion in cancer therapy to overcome multiple drug resistance of solid tumors.

***Keywords:*** *Targeted drug delivery, Nano particle, Chitosan, Nano capsulated system, Liposomes*

## 1. INTRODUCTION

Intended for the targeted population, the route of drug delivery is to ensure that it can manage the illness and provide therapy. But at present time the conventional technique of drug delivery system itself is the main issue, the medicine does not reach the target because they cannot pass the micro-capillaries. Another method is the use of liposomes as possible carriers has one of the most interesting advantages – how pharmaceuticals can be delivered and protected from destruction by the body, concentrated at the place of action, and minimized the toxin effect on other organs. However, several disadvantages such as low EE%, rapid release of water-soluble drugs in presence of blood components, and poor storage stability of liposome-based formulation have prompted the present investigation for a better system than liposomes. Therefore, the delivery processes substitute nanoparticles, which are particulate dispersions or solid particles with a size at the submicron level from 1-100 nm and can be used as matrix to facilitate the transport of the therapeutic lead molecule either dissolved, entrapped or attached.With these nanoparticle based drugs, the approach to the cancer treatment can be greatly enhanced. Nano particles could either be nano-spherical or nano-capsule formations according to the type of preparation method adopted. Nano capsulated systems are the systems where drug is confined at the center of the system and surrounded by a polymer barrier. The nanospheres are matrix systems through which homogenous drug dispersions are produced. At present, complex and multiple-dimensional nanotechnology platforms in the developmental or clinical phase are being studied to design better and safer drugs with less side effects.

It is agreed that polymers are the most suitable materials for fabricating numerous molecular patterns to be incorporated as different nanoparticle constructions for various biological applications, especially in dealing with cancer. Researchers have described intravenous injections. Polymeric nanoparticles prepared from biodegradable polymers like poly?DL-lactic acid (PLA)/poly caprolactone (PCL), are employed as drug delivery devices to deliver the drug to a specific organ provided the nanoparticles are coated with hydrophilic polymers like PEG.

It has been described that polymeric nanoparticles have replaced the aforementioned liposome method because of better drug/protein stability and long period drug release. Polymeric nanoparticles' size explains how long the medication will be retained in the bloodstream. There was a previous work done that employed a sterically stabilized ligand nanoparticle system to deliver antisense oligodeoxynucleotides, and small interfering RNA to lung cancer cells. Therefore, the syntactic idea in this study is clearly evident, proving that the carrier or the delivery system via a polymeric nanoparticle is highly achievable and target specific.

Chitosan is a non-toxic biological product polymer [33, 34], derived from partial deacetylation of chitin, which is found in crustacean and insect shells. It consists of glucosamine and N-acetylglucosamine repeating units and the amounts determine the extent of polymer deacetylation. While chitosan is insoluble and has a neutral charge at near physiological pH, it is soluble and a positively charged polymer at low pH. Chitosan can be of high molecular weight, low molecular weight and more. Compared to high-MW chitosans, L-MW and L-DA chitosann have higher solubility and faster degradation profiles. Studies also reveal that chitosan has antimicrobial properties, antifungal activity and exhibits property of wound healing. Another study, employing chitosan to determine the LD, reported an oral LD50 above 16 g/kg of the body weight in mice, and therefore, Chitosan is non-toxic, and more importantly biodegradable.

Sodium alginate is composed of alginic acid and D-mannuronic acid and L-guluronic acid residues connected by − 1, 4 and − 1, 4 glycosidic Linkages. Gelatin is a biopolymer with good biomedical compatibility and biodegradable properties suitable for pharmaceutical and medical uses. In an earlier identification, 100 nm gelatin particles after delivery were observed to target the leaky tumor vasculature and did not penetrate the dense collagen matrix of the interstitial space. In addition, the MMP-2 activity removed the gelatin core of the 100 nm particle, and the 10 nm particles on the surface appeared from the outside. Due to their relatively diminished size, these MMP-2 modified particles have the ability to diffuse considerably to the interior of tumors. Two of the compounds identified as having therapeutic use are Isolongifolene (ILF), a carbazole alkaloid extracted from curry leaf plant, specifically from the plant called; Murraya koenigii, an Indian herb. Isolongifolene has a woody and amber incense-like note, and is used in cosmetics, perfume, space spray, soaps, detergents, deodorants and in materials. Isolongifolene is a commercially available and is a sesquiterpene hydrocarbon, having isolongifolene framework. In an in-vitro model of Parkinson's disease (PD), it has been shown that ILF provides neuroprotection against rotenone-induced neuropathogenic changes including oxidative stress, mitochondrial dysfunction, and apoptosis. In a rat model of rotenone-induced PD in this study, ILF was reported to enhance the behavioral deficit and decrease the oxidative stress. Therefore, given the mucosal expression of ILF, we decided that this protein may be a pharmacological target for the treatment of the disease. Consequently, there is a necessity for a nano-sized particle delivery system of medicine to ensure that medicinal lead delivers into the host system. In this study, we have designed, developed and fully characterised a polymeric nano formulation using isolongifolene for additional advantages and have also determined the compatibility involving polymers including sodium alginate, chitosan and gelatin. The therapeutic efficacy, stability and cytotoxicity of the Nano formulations for cancer therapy were also compared with the improved formulations. Therefore, our study aimed at characterizing isolongifolene-loaded polymeric nanoparticles, which have excellent adjuvant effects and can be applied to multiple diseases.

## 2. MATERIALS AND METHODS

**Materials.** Isolongifolene, Chitosan, and Sodium tri poly-phosphate were procured from Sigma Aldrich (USA). All other chemicals used in this study were of analytical grande.

**Fabrication of isolongifolene loaded chitosan nanoparticles:**

The Isolongifolene-loaded Chitosan Nanoparticles (ICN) were prepared by ionic cross-linking chitosan with sodium tripolyphosphate (TPP) anions, following the method reported by our group. Namely, chitosan solution of 2 mg/mL was prepared in 0.25% (v/v) acetic acid solution at 10 °C under stirring for 12 h. About 0.75% w/v of TPP (aqueous solution) was added (2:1 ratio) in to the chitosan solution containing 100 µg/mL of isolongifolene dissolve in methanol with continuous stirring for 6 h at 4 °C. The subsequent dispersion was subjected to centrifugation at 13 000 × g for 20 min at 4 °C. This ICN was further concentrated by pelleting the cells followed by removal of the supernatant and aliquoting the material was stored at − 55 °C.

**FT-IR analysis:**

The ICN was further described using the FTIR spectroscopy to determine the molecular interconnection of isolongifolene and the chitosan polymer matrices. The FTIR scanning range was set between 4000 and 400 cm−1 cm.

**X-Ray diffraction study:**

In order to further support the claimed amorphous structure of ICN, X-ray diffraction analysis was carried for chitosan also, as well as for ICN. This experiment was done by using Cu-Kα1 radiation of 40 kV and 30 mA. The scanning rate was 5°/min for a scan sector of 4–90°with step, 0.1°.

**Optimization of ICN:**

Different preparations of ICN nano were tried by varying the proximate ratio of CH significantly to a constant amount of isolongifolene. The different chitosan concentrations of 0.1, 0.2, 0.3, 0.4, and 0.5 % w/v were obtained by dissolving chitosan in acetic acid solution (0.25% v/v) under agitation (250 rpm) for 24 hours. The chitosan solution (10 mL) containing 0.20 mg of isolongifolene was mixed with about 20 mL of TPP of 0.75% (w/v) under constant mixing at 320 rpm for six h at four °C. Sonication was performed at four °C using Sonitvibra cell UC130, USA; amplitude 20 and pulse 4s for 10 min. Then, the dispersion was washed three times (2200 × g) with water LiChrosolv HPLC grade and re-dispersed. Further, the resultant solution was centrifuged at 13,000 × g for 20 min at four °C to obtain five different Isolongifolene polymer ratio nano formulations (ICN-K01 (1:0. These were also found to be in the ratio of 1: 5, 1: 1, 1: 1.5, 1: 2, 1:2.5 as depicted in figs. ICN-K02, ICN-K03, ICN-K04 and ICN-K05. The prepared nanoparticles were then lyophilized (Delvac-lyo1550, INDIA) and preserved at 20° C for characterization analysis.

**Process yield:** The process yield was established by weighing the freeze-dried nano formulations, which were the ICN-K01 to the ICN-K05, as described in the described method. All process yield data percentages are given as the mean of three replicates of the process yield.

**Calculation of drug encapsulation efficiency (DEE) and drug loading efficiency (DLE).** The DEE and DLE were determined using the method that has already been described with slight modifications. Additionally, ICN was pelleted at 5800 x g and the pellet containing the organelles of interest was washed and quantified for isolongifolene by HPLC as done before. The percentages of DEE and DLE were calculated using the formula given below: -

$$\text{DEE} = \frac{Totla\ amount\ of\ drug - drug\ in\ supernantant}{The\ total\ amount\ of\ drug}\ X\ 100$$

$$\text{DEE} = \frac{Totla\ amount\ of\ drug - drug\ in\ supernantant}{Weight\ of\ recovered\ nanoparticles}\ X\ 100$$

**In vitro drug release study of isolongifolene:**

In vitro drug release was investigated by plasma simulation and dialysis membrane method with some changes. The nano formulations were redispersed in 10 ml of 0.9% saline containing Isolongifolene at a final concentration of 20 µg/mL. To 1 mL of the above prepared mixture, 10 mL of 0.5M PBS, pH-5.5 and 10 mL of plasma were added. The suspension was placed in an orbital shaker at 37 °C. A volume of one millilitre of the released solution is collected at different period of time and replenished in the process. Isolongifolene content of the harvested solutions was determined using the HPLC method after the solutions had been centrifuged and the supernatant was used for isolongifolene quantification.

$$\text{Isolongifolene (\%)} = \frac{Amount\ of\ Isologifolene\ released\ at\ a\ time}{The\ amount\ of\ Isologifolene\ loaded\ in\ the\ nano\ formulation}\ X\ 100$$

In the dialysis membrane method, 2 mg of nanoformulations were redispersed in 10 mL of PBS (0.5 M, pH = 5.5), placed in a dialysis membrane (cut-off 10 kDa) and dialyzed against PBS (0.5 M pH = 5.5). 2 mL of released solution was collected at different time intervals, replaced with fresh solution and analyzed using HPLC. The release rate of Isolongifolenecan is derived from the calibration curve prepared using known concentrations.

**Size, shape, and zeta potential measurement:**

The size distribution and zeta potential of different nanoformulations (ICN-K01–ICN-K05) were measured by Zetasizer Nano ZSinstrument (Malvern, Mastersizer 2000, UK). The sample was dispersed in water (pH = 5.5) and the nanoparticles were counted in a 4.8 mm calibrated area with a count rate of 210.3 kcps (kilo counts per second) for 70 s. The average hydrodynamic diameter of different nanoformulations was calculated as mean values. The surface morphology of ICN was analyzed using Field Emission-Scanning Electron Microscopy (FE-SEM) (TESCAN, VEGA3 SBU, Czech). The average particle size and shape of ICN were further studied using High Resolution-Transmission Electron Microscope (HR-TEM) (Jeol, JEM2100, Japan).

**Stability of ICN in blood plasma:**

Fresh blood from Wistar rats was collected in heparinized tubes and the plasma was separated by centrifugation (1800 × g for 15 min at 4 °C). The 10 µg conc. of ICN-K04 was added into the separated plasma and further incubated at 37 °C for 30 min in 0.9% (w/v) of NaCl solution. 1 mL plasma solution was collected at regular time intervals and stored at − 20 °C until use. The isolongifolene content was analyzed by the HPLC method after centrifugation at 17,000 × g for 20 min.

**Hemocompatibility study:**

The hemocompatibility of ICN was evaluated using the reported procedure. The whole blood was collected from a Wistar rat and anticoagulated with sodium citrate (ratio of blood to anticoagulant taken was 9:1). Erythrocytes were isolated by centrifuging whole blood at 1000 × g for 10 min. The erythrocytes were washed thrice with saline before use. ICN-K04 was mixed with RBCs in different concentrations (2–24 µg/mL) and then incubated for 2 h at 37 °C and the supernatant was collected by centrifugation at 1500 × g for 5 min. Hemoglobin release was monitored spectrophotometrically (Systronics,

2203, INDIA) at 541 nm.

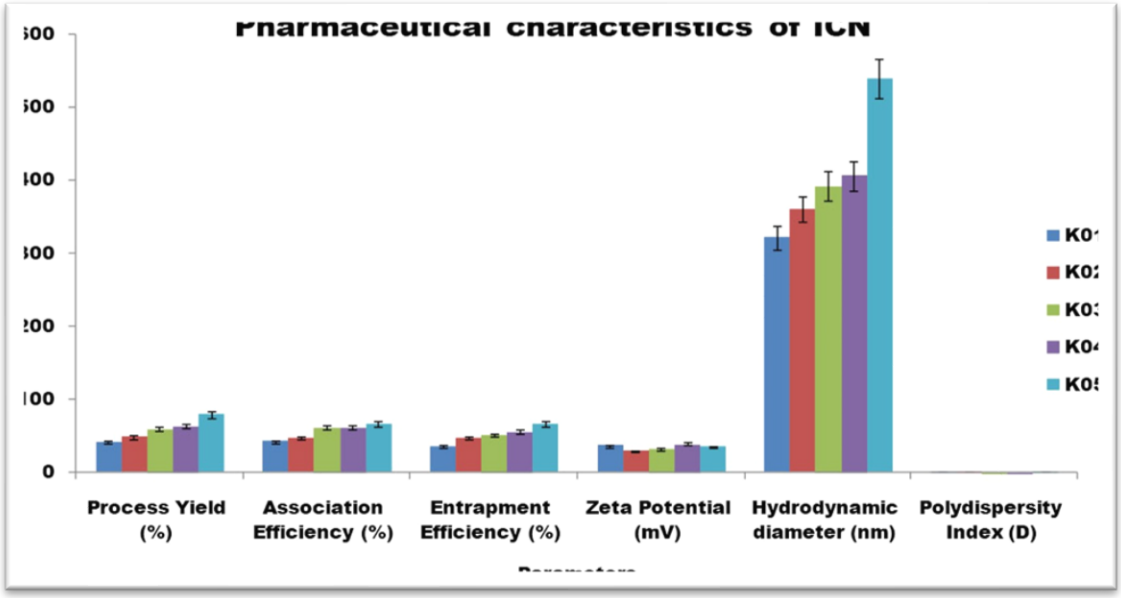


**Figure 1. Statistical representation of pharmaceutical characteristics of ICN.**

The TritonX-100 (1% v/v) and 0.9% (w/v) NaCl were taken as positive and negative controls respectively. The percentage of hemolysis was calculated using the following formula:

**Percent hemolysis** = (O.D. Test / O.D. Sample) x 100

**Measurement of cell viability.** Cell viability was analyzed using a conventional MTT reduction assay. Cells were treated with ICN and the viability was assessed based on the detection of mitochondrial dehydrogenase enzyme activity in viable cells. Cells were cultured in a 96-multiwell plate. The 3 × 103 cells were seeded to each well. Initially, the cells in the medium were pre-incubated with or without ICN for 24 h. After 24 h, the cells were incubated with MTT (5 mg/mL) at 37 oC for 4 h. Following incubation, the medium was removed and the formed formazan crystals were dissolved with DMSO. The absorbance of the reduced product, formazan was measured at 570 nm using an ELISA plate reader (Bio-Rad, Hercules, CA, USA). The percentage of cell viability can be determined using the below formula: -

$$\text{Cell viability} = \frac{O.D.of\ control - O.D.of\ test\ compund}{O.D.of\ control}\ X\ 100$$

where O.D represents Optical Density.

**Apoptosis study.** The A549 cell lines were procured from ATCC, USA and were used for apoptotic studies. The Apoptosis assay was performed using both acridine orange (AO) and ethidium bromide (EtBr) dyes. Acridine orange, a permeable dye stain all the cells, and ethidium bromide permeate into the cell only when the cell membranes disintegrate. EtBr intercalates with DNA forming an orange-red complex. Once the medium was removed from the plates after treatment, the cells were washed twice with phosphate-buffered saline (PBS) and stained with AO and EtBr dyes. The stained cells were incubated for 20 min at room temperature and washed with warm PBS to remove the excess dye. The cellular morphology was observed using a fluorescent microscope (λex/λem = 490 nm/530 nm) and the images were captured. The fluorescent intensity was recorded at 535 nm using Spectro-fluorimeter.

## 3. RESULTS

**FT-IR analysis:** The FT-IR spectrum of isolongifolene polymers such as sodium alginate, gelatin, and chitosan and their interaction with isolongifolene were shown in Fig. 1. The FTIR spectra of isolongifolene shows signature peaks at 1242 cm−1 (C–O stretch), 1407 cm−1 (C–C stretch), 1590 cm−1(C=O stretch), 2965 cm−1(C–H stretch), 3688 cm−1(O–H stretch). ISN spectrum showed significant peaks at 3218 cm−1(O–H vibration) and 1673 cm−1(C=C stretching). However, an ISN spectrum lacks an FT-IR band at 1242 cm−1corresponding to the C–O stretch of isolongifolene (Fig. 2).

## 4. DISCUSSION

Hitherto, the major clinical challenge in treating the cancer is the relapse prevalence, which occurs due to the failure of the primary treatment regimen in targeting the cancerous cells. Therefore, drugs having the potential of huge accessibility and

specifically delivered to the target site hold a great promise in the management of cancer. Nanoparticle-based drug delivery system has gained huge attention due to its versatile approach to accessing the cancerous site and another inflammatory milieu. Thus, the synthesized nanoparticles have a unique potential to overcome the poor penetrating ability and also exert their maximum efficacy to control the proliferation of the disease.

The current study investigated the compatibility of Isolongifolene nanoformulation with different polymers. Preliminary studies showed that the chitosan-based Isolongifolene polymeric nanoformulation could act as an excellent adjuvant in therapeutics, mainly treating multi-drug resistance in solid tumors.

In the FT-IR spectroscopic study, the peaks revealed the surface chemistry of the presence of functional groups other than the innate molecules as confirmed by the signature FT-bands with different wavenumbers corresponding to different significant changes obtained in shape and position of the absorbance bands. Among the different ratios investigated, the 1:2.5 ratio of isolongifolene and polymer ratio yields a higher process yield, which could be due to greater carbon efficiency. In an experimental condition reduction of pH leads to greater association and loading efficiency.

Zeta potential is an important physicochemical property that reflects the physical stability and mucoadhesive properties of nanoparticles. In principle, zeta potential values in the range of <− 30 mV and > + 30 mV are considered stable regimes. In the present study, the ICN-K04 formulation possesses a zeta potential of +39 mV

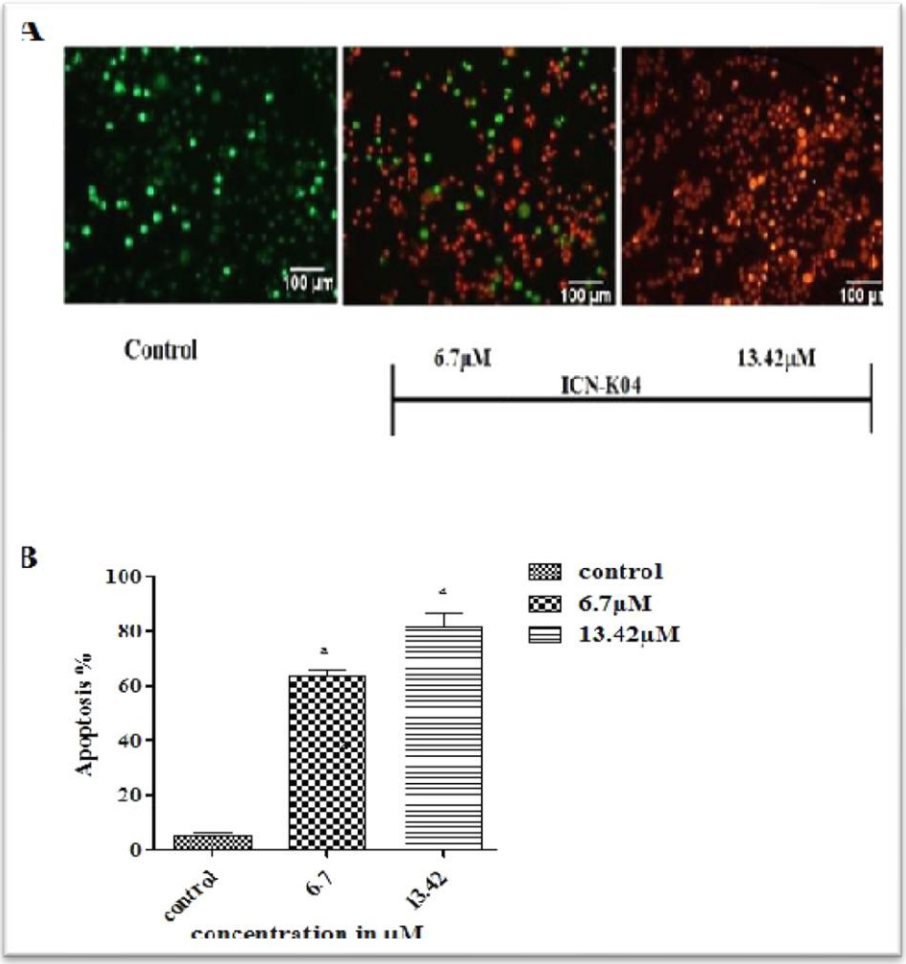


**Figure 10. Apoptotic effect of ICN-K04. (A) Photomicrograph showing the effect of ICN-K04 induced apoptotic morphological changes in A549 cells. (B) Changes in fluorescent intensity after treatment with ICN- K04 in A549 cells as compared to control cells after 24 h.**

Values are expressed as Mean ± SEM. $P < 0.05$. among others, which demonstrates its good interaction stability. Polydispersity Index (or) PDI is the measure of the particle size distribution with values ranging from 0 to 1. In this study, the PDI values close to 0 (zero) presented a homogeneous dispersion and those greater than 0.5 showed high heterogeneit. The prepared nano formulations (ICN-K01 to ICN-K05) showed PDI values in the range of 0.1 to 0.4. Especially, ICN-K04 possesses a PDI of 0.1 which indicates the presence of monodispersed (homogenous) particles.

Moreover, the nanoformulation ICN-K04 showed increased association efficiency which was inversely proportional to the isolongifolene release. Subsequently, in vitro release of isolongifolene monitored by plasma simulation and membrane dialysis method showed the lack of burst effect and also confirmed that the interactions between isolongifolene and the nanoparticles were weak. ICN formulation was found to be optimum based on zeta potential, PDI value, and in vitro release methods, which demonstrate that ICN-K04 possesses good stability and homogenous dispersity. Further, the ICN-K04 formulations were used for the anti-carcinogenic study.

The ultra-structural image of the formulation revealed the presence of nearly shaped solid particles after the incorporation of isolongifolene. The formation of intermolecular hydrogen bonding did not show any significant shearing effect on the surface of ICN-K04. The biocompatibility of nano formulations used as drug delivery systems is a crucial parameter. The integrity of the hemoglobin structure might function as a key factor to determine the potential biocompatibility of nanoparticle. A few other mechanisms have been proposed whereby hemolysis can contribute to thrombosis in addition to changing the hematocrit and hemorheology, such as the release of erythrocyte-derived macrovesicles, activation of the complement cascade, and the release of free haemoglobin and heme into circulation, which sequesters nitric oxide. Hemolysis is a crucial factor in the hemocompatibility testing of biomaterials and can have a big impact on how well they work in the clinic. A number of NPs, including amorphous silica, tricalcium phosphate, hydroxyapatite, and particularly silver (Ag) NPs, have been discovered to significantly cause hemolysis, endangering their use in biomedical applications.

Most NPs have hemolytic activity; however, it depends on concentration, structure, size, and shape. For instance, the amount of reactive silanol groups exposed on the surface of silica NP is directly related to the size and geometry of the NP. Surface charge, shape, porosity, and surface functionalization with certain polymers or functional groups are the most important surface characteristics that determine the hemocompatibility of NPs. Upon administration, injected particles will most likely interact with red blood cells. Electrostatic interactions between the red blood cells and the nanoparticles can cause perturbation of the membrane, thereby causing hemolysis. In the present experimental study ICN-K04 showed and induced hemolysis at higher concentrations, which could be due to the electrostatic attractive forces between chitosan and erythrocytes and subsequently lead to thrombus formation. Nanoparticle movements were faster as compared to macromolecules. It was also expected that the nano formulations could reach the tissue compartment within 50 min.

In cancer therapy, tumor growth can be suppressed by activating the apoptotic machinery in the cell. Many malignant cells, however, are unable to regulate the genes that control apoptosis, rendering them resistant to the induction of apoptosis by a variety of stimuli, including intracellular and extracellular signals chemotherapeutic drugs, and radiotherapy.

A previous study has investigated that the SH-SY5Y cells exposed to rotenone caused about 50% cell death, whereas isolongifolene pre-treated cells dose-dependently regulated the toxic effects of rotenone by which it exhibits neuroprotective effect against apoptosis. In our study, we investigated the cytotoxicity of nano formulation ICN-K04 at different A549 cell lines for 24 h. The result exhibited that the viability of cancer cells was inhibited by 50% in a dose-dependent manner, and also, we found that 50% inhibitory concentration (IC50) of ICN-K04 at 13.42 µM among all other concentrations respectively.

## 5. CONCLUSION

Isolongifolene loaded chitosan nanoparticles were successfully prepared and characterized for parameters like association efficiency, loading efficiency, zeta potential, entrapment efficiency, and polydispersity index (PdI). Among the nano formulations (ICN-K01to ICN-K05) prepared, the ICN-K04 formulation was found to be optimum. The X-ray diffraction spectrum of ICN-K04 formulation confirms the poor crystalline nature of the nanoparticles. The spherical morphology of ICN-K04 nanoparticles was confirmed by scanning and transmission electron microscopy. More importantly, ICN-K04 nanoparticles have low hemolytic activity and also lack genotoxicity. ICN-K04 nanoparticles show intrinsic plasma stability of more than 50 min. As a prerequisite for a nanoparticle-based drug delivery system, it is imperative to understand the size, morphology, as well as surface charge of nanoparticles which can deliver the loaded drug effectively and specifically to its targeted site. From the results it can be corroborated that the ICN-K04 nanoparticles show promising data in all the physiochemical criteria, so that they can be used as a delivery system for the drug to show conspicuous effects to the targeted site. Further, validation studies of this drug delivery approach using the nanoparticles system are underway.

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