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Preparation and Characterization of Sodium Alginate Nanoparticles Containing ICD-85 (Venom Derived Peptides)

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ABSTRACT: Sodium alginate is one of such biodegradable polymers, which has been extensively exploited for the preparation of nanoparticles (NPs) for controlled delivery of several therapeutic agents. ICD-85 (venom derived peptides) has been shown to exhibit anti-cancer activity. In this report sodium alginate nanoparticles employed to improve upon its effectiveness. ICD-85 loaded NPs were prepared by ionic gelation method and were characterized by the particle size, zeta potential, transmission electron microscopy, FT-IR spectroscopy and in vitro release studies. The in vitro cytotoxicity was evaluated by MTT assay. TEM revealed ICD-85 loaded NPs to have spherical shapes with a size of approximately 200 nm. The zeta potential of the ICD-85 loaded NPs was estimated as –16.1 mV. Loading capacity and encapsulation efficiency were 90.48% (w/w) and 90.24% (w/w), respectively. The in vitro release profile exhibited sustained release patterns with relatively initial burst release, followed by a subsequent slower release. Cytotoxicity assay showed that ICD-85 loaded NPs is more potent than free ICD-85 in suppressing proliferation of HEp-2 cells. In conclusion the ICD-85 loaded NPs presented high loading capacity and sustained release profile which effectively inhibit the proliferation of HEp-2 cell line in vitro, and may be a beneficial agent against human carcinoma.

KEYWORDS: ICD-85, Nanoparticles, Encapsulation, Sodium alginate, Ionic gelation, HEp-2 cell line.

1 INTRODUCTION

Nanoparticles (NPs) have become a focus of attention in the field of biomedicine owing to their capacity to deliver various drugs [1]. Much research has been focused on the preparation of nanoparticles using biodegradable hydrophilic polymers such as alginate and chitosan [2], [3]. Alginate has been found increasing biotechnological and biomedical applications in view of its several advantages, such as high biocompatibility, biodegradability, non-toxicity, non-immunogenicity, chelating ability, and the possibility of chemical modification [4], [5], [6], [7]. Moreover, alginate has been widely used for encapsulation of cells [8], [9], proteins [10], DNA [11], venoms [12] and vaccines [13]. Recently, alginate was developed as a nanoparticle for the oral delivery of insulin [14]. On the other hand, alginate has already acclaimed permission from US FDA [4].

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Nanoparticles have been prepared using several different methods [15]. One method for preparing nanoparticles based on polysaccharides is the ionic gelation method [16]. This method offer many advantages such as simple and mild preparation method without the use of organic solvent or high shear force [17], [18], [19].

One of the major problems facing cancer chemotherapy is the achievement of the required concentration of the drug at the tumor site for a desired period of time, since tumors usually present resistances to treatment, and high dosages are frequently toxic [20], [21]. Thus, one of the main goals of nanomedicine is to develop safe and effective drug carriers that are systemically applied but will selectively deliver cytotoxic drugs to tumor cells without harming normal cells [22], [23]. Polysaccharide-based NPs play an important role and their use with some anti-cancer drugs show promising results [24], [25], [26], [27].

Our previous studies revealed an inhibitory effect of ICD-85 (venom derived peptides) on MDA-MB231 and HL-60 cancer cell lines through induction of apoptosis [28], [29]. ICD-85 was also confirmed by in vivo studies to suppress the breast tumor in mice [30].

In this study we attempt, to prepare sodium alginate NPs and encapsulated ICD-85 as cytotoxic agent to evaluate the usefulness of nanoparticles as a carrier of ICD-85 by measuring the in vitro cytotoxicity of nanoparticles on the proliferation of HEp-2 cell line.

2 MATERIALS AND METHODS

2.1 MATERIALS

The active fraction of ICD-85 is a combination of three peptides, ranging from 10,000 to 30,000 Da, derived from the venoms of snake (*Agkistrodon halys*) and scorpion (*Hemiscorpius lepturus*) was obtained from Razi Vaccine and Serum Research Institute (Karaj, Iran). The cell culture medium (RPMI 1640), fetal calf serum (FCS), trypsin–EDTA, penicillin and streptomycin were provided by Gibco (USA). Sodium alginate and poly-L-lysine (PLL) were purchased from Sigma-Aldrich Chemical (Germany). Calcium chloride, dimethyl sulfoxide (DMSO) and 3-(4, 5-dimethyl-thiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) were purchased from Merck (Darmstadt, Germany). All other chemicals used in this study were of analytical grade.

2.2 PREPARATION OF NANOPARTICLES

2.2.1 PREPARATION OF SODIUM ALGINATE NPS

Sodium alginate NPs were prepared by ionic gelation method adapted from Rajaonarivony's method of preparing alginate–poly-L-lysine nanoparticles [16]. Initially, 1 ml of calcium chloride was added to 19 ml of sodium alginate solution to induce gellification. Then 8 ml of poly-L-lysine was added in order to condensation of nanoparticles. The nanoparticles suspension obtained was stirred for 2 h and kept overnight for stabilization. The nanoparticles were separated by centrifugation (Sigma, USA) at 13000 rpm for 30 minutes, freeze-dried and stored at 4°C. The weights of freeze-dried nanoparticles were also measured.

2.2.2 PREPARATION OF SODIUM ALGINATE NPS CONTAINING ICD-85

The concentration of sodium alginate was fixed at 0.3 mg ml^{-1}, whereas the concentration of calcium chloride was 0.2 mg ml^{-1}. The ICD-85 loading nanoparticles were prepared with incorporation of sodium alginate solution into calcium chloride solution containing 334 µg ml^{-1} of ICD-85.

2.3 CHARACTERIZATION OF NANOPARTICLES

2.3.1 PARTICLE SIZE ANALYSIS AND ZETA POTENTIAL MEASUREMENT

The average particle size and size distribution of ICD-85 loaded NPs were determined by dynamic light scattering (DLS), using Malvern Zetasizer (Malvern Instruments, UK) with a wavelength of 532 nm at 25°C with an angle detection of 90°. In brief, 1 mg ml^{-1} of nanoparticulate suspension was prepared in double distilled water and sonicated for 1 min over an ice

bath. Then, 0.1 ml of the above NPs suspension was diluted to 1 ml in water and then subjected to particle size measurement.

The zeta potential was measured by the same instrument. Measurements were made at 25°C without sample dilution or any salt addition. All measurements were performed in triplicate.

2.3.2 TRANSMISSION ELECTRON MICROSCOPY (TEM)

Specimens were prepared by dropping the sample solution onto a copper grid. The grids were subsequently negatively stained with 2% phosphotungstic acid solution. The grid was then allowed to stand for 30 s to 1 min before the excess staining solution was removed by draining. The specimens were air-dried and examined using a Philips 400 transmission electron microscope (Netherlands) at an accelerating voltage of 80 kV.

2.3.3 FT-IR SPECTRUM ANALYSIS

ICD-85 loaded NPs were separated from the suspension by centrifugation at 13,000 rpm and 14°C for 30 min and lyophilized. These dried nanoparticles were mixed with KBr and pressed to the plate for measurements. FT-IR spectra were recorded on FT-IR spectrometer (FTIR- 410® Jasco Colchester, UK).

2.3.4 ENCAPSULATION EFFICIENCY AND LOADING CAPACITY

ICD-85 loaded NPs were separated from aqueous suspension by centrifugation at 20,000 rpm and 14°C for 30 minutes. The supernatant was collected and protein content (ICD-85) in supernatant was determined by the Bradford protein assay spectrophotometric method at 595 nm [31]. The ICD-85 encapsulation efficiency (*EE*) and loading capacity (*LC*) of nanoparticles were calculated by using following equation:

$$\%EE = [(A-B)/A] \times 100$$

$$\%LC = [(A-B)/C] \times 100$$

A = total amount of ICD-85 in added solution; *B* = total amount of ICD-85 in supernatant after centrifugation; and *C* = weight of the nanoparticles measured after freeze-drying [32].

2.4 IN-VITRO RELEASE STUDY

In-vitro release of ICD-85 from NPs was carried out by dispersing 100 mg of NPs in 10 ml of PBS (0.01 M, pH 7.4). The nanoparticulate suspension was equally divided in ten tubes. 1 ml in each and kept in a shaker at 37°C and 300 rpm. At particular time intervals (2, 4, 6, 12, 24, 36, 48, 60 hours) one tube was removed and the sample was centrifuged at 13,000 rpm and 14°C for 30 minutes. The amount of ICD-85 released in the supernatant was measured.

2.5 CELL CULTURE AND TREATMENT

The human larynx carcinoma cell line (HEp-2) obtained from cell bank of Razi Vaccine and Serum Research Institute (Karaj, Iran). Cells were cultured in RPMI 1640 medium supplemented with 10% FCS, streptomycin (100 µg ml^{-1}) and penicillin (100 IU ml^{-1}) in 25 cm^2 culture flasks (Nunc, Denmark) at 37°C in 5% humidified CO2 incubator (Memmert, Germany). For subculture, cells at 80–90% confluence were passaged at a ratio of 1:3 after detachment with 0.25% trypsin containing 0.02% EDTA [33].

Experiments were carried out 24 h after cells were seeded. The cultured cells were treated with free ICD-85 and ICD-85 loaded NPs and examined their effects in different concentrations. The cells were evaluated after 72 h of the treatment.

2.6 IN VITRO CYTOTOXICITY ASSAY

Cell proliferation was determined by MTT colorimetric assay [34]. MTT-based in vitro cytotoxicity assay was performed to compare anti-cancer effects of ICD-85 loaded NPs versus free form of ICD-85 on HEp-2 cell lines. Briefly, cells in logarithmic growth were seeded at a density of 5×10^5 cells ml^{-1} in 96-well culture plates (Nunc, Denmark) in 0.2 ml^{-1} volume media. After an overnight incubation, cells were then treated with different concentrations of free ICD-85 and ICD-85 loaded NPs (0, 1, 5, 10, 20 and 30 µg ml^{-1}). After 72 h of the treatment, 0.02 ml^{-1} of MTT solution (5 mg ml^{-1} in PBS) was added into each well and

cells were incubated at 37ºC for 4 h allowing the MTT to be metabolized. After incubation, purple crystals were observed and the media was removed from each well by aspiration. The crystals were then dissolved by adding 0.2 ml^{-1} of DMSO to each well. DMSO was also added to the wells designated as reference blanks. Then, the optical density of 96-well plates was measured using an ELISA reader (Dynex MRX II, USA) at 570 nm. The optical density of untreated control cells was taken as 100% of viability. Statistical analysis was done to determine the means ± SD of cell viability. The percentage of surviving cells was calculated as the percentage of MTT absorption according to the following formula:

% Survival = (mean experimental absorbance/mean control absorbance×100).

2.7 Statistical Analysis

Computer program (Graph Pad Prism) was used to calculate the IC_{50} (50% inhibition of cell proliferation) values. All experiments were repeated at least three times. Statistical analyses were performed using a Student's t-test and the results were presented as mean ± SD. Statistical significance was accepted at a level of $p<0.05$.

3 Results

3.1 Physicochemical Characterization of Nanoparticles

The morphological characteristics of nanoparticles were examined using TEM. The TEM image of nanoparticles was shown in figure 1, which revealed relatively smooth and spherical nanoparticle structures. The mean hydrodynamic diameter of particle was approximately 300 nm having polydispersity index (PDI) 0.432.

The zeta potential of the sodium alginate NPs was estimated –21.9 mV and changed to –16.1 mV after loading with ICD-85.

The FT-IR spectroscopy was carried out to study the possibility of chemical interaction between ICD-85 and sodium alginate was shown in figure 2. The band around 3300–3400 cm^{-1} range in the spectrum contributes O–H stretching and intermolecular hydrogen bonding. The peaks observed at 1400–1700 cm^{-1} in the spectra represent peaks belong to the C=O stretching (amide). The characteristic peak observed around 1600 cm^{-1} (salt of carboxyl group) in the FT-IR spectrum of nanoparticles was attributed to the ionic interaction between these two reactive groups.

Loading capacity and encapsulation efficiency were 90.48% (w/w) and 90.24% (w/w), respectively.

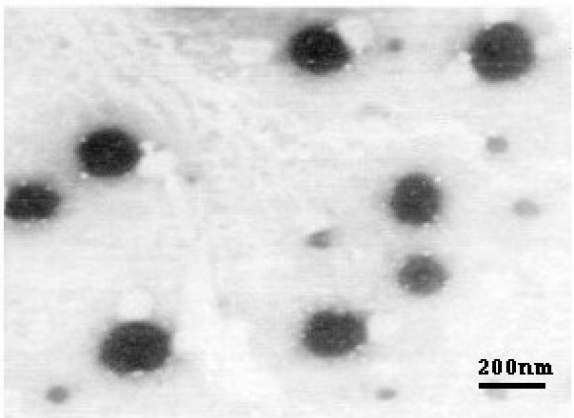

Fig. 1. TEM image of ICD-85 loaded sodium alginate NPs

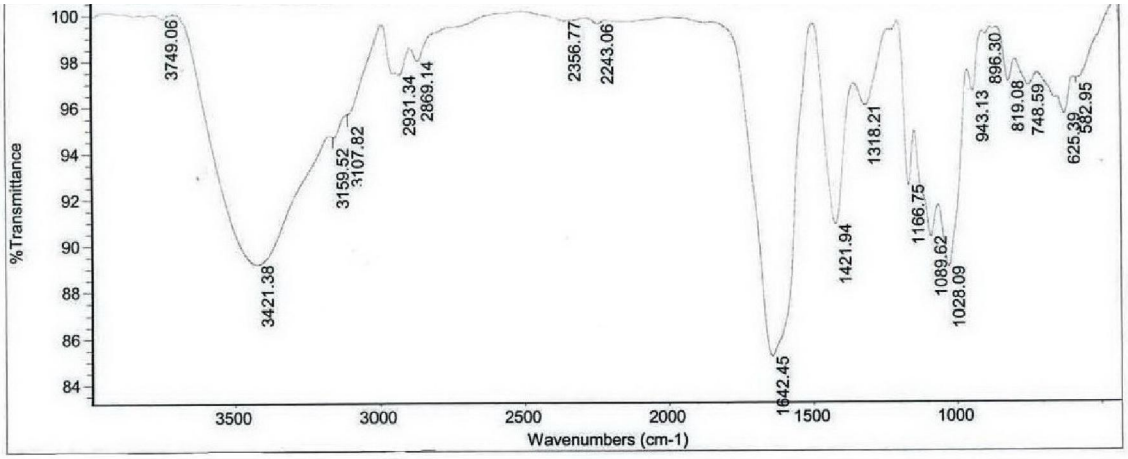

Fig. 2. FT-IR of ICD-85 loaded sodium alginate NPs

3.2 In Vitro Release Study

The in vitro ICD-85 release data are presented in figure 3. ICD-85 loaded NPs showed 7% and 31% release of ICD-85 in 2 h and 24 h, respectively. As shown in the ICD-85 loaded NPs release profile after 60 h, about 90% of the ICD-85 released from the nanoparticles.

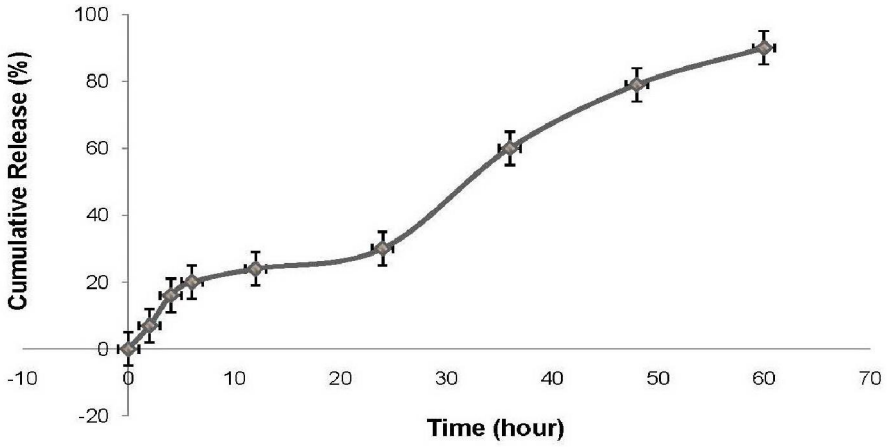

Fig. 3. In vitro release profile of ICD-85 loaded sodium alginate NPs

3.3 In Vitro Cytotoxicity

Figure 4 shows the cell viability treated with free nanoparticles, free ICD-85 and ICD-85 loaded NPs at various concentrations for 72 h incubation time. The free nanoparticles did not demonstrate any significant effect on the viability of HEp-2 cells. The viability of cells treated with free ICD-85 and ICD-85 loaded NPs decreased with increasing concentration. We compared the ability of free ICD-85 and ICD-85 loaded NPs to inhibit the proliferation of HEp-2 cells. ICD-85 loaded NPs inhibited the proliferation of HEp-2 cells in a dose-dependent manner with an IC_{50} value of 5.2 µg ml^{-1} as compared to IC_{50} of 11.1 µg ml^{-1} for free ICD-85.

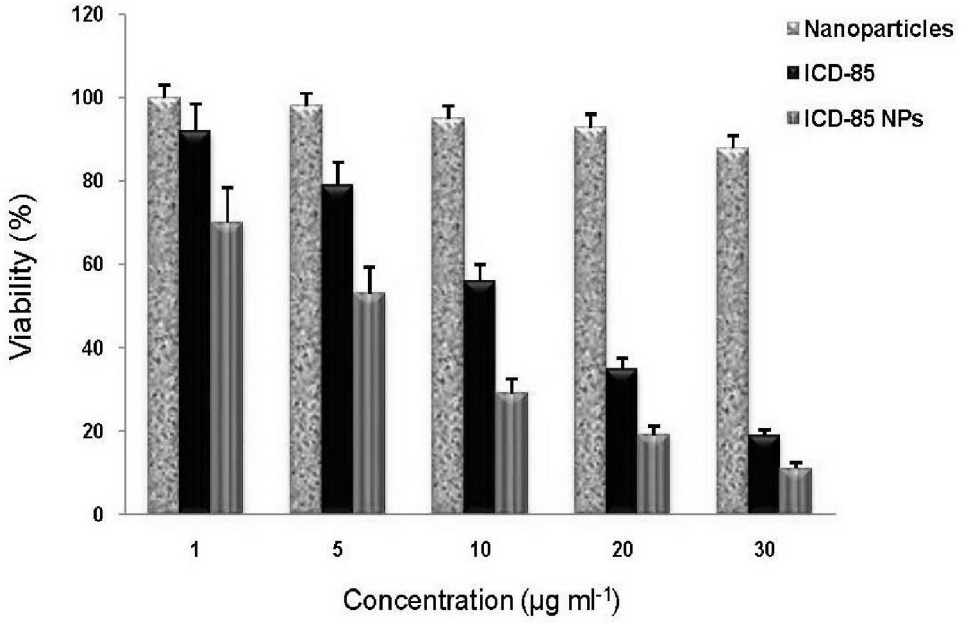

Fig. 4. Viability HEp-2 cells exposed to free nanoparticles, free ICD-85 and ICD-85 NPs at 72 h and measured by MTT assay

Viability of untreated cells (0µg) was taken as 100%. The measurements of the treated cells were normalized to the control measurement (100%). Results are expressed as mean ± SD (n=3 experiments, six replicates per experiment at each test concentration). Significant difference ($P<0.05$, Student's t-test) in proliferation of HEp-2 cells was observed between free ICD-85 and ICD-85 loaded NPs.

4 DISCUSSION

In recent years, a large number of studies have been conducted on polysaccharides and their derivatives for their potential application as nanoparticle drug delivery systems [24], [25], [26], [27]. Modern developments in polymeric nano-formulations for the treatment of cancer have reported for sustained and controlled delivery of anti-cancer agents [35], [36], [37], [38].

The current studies were designed to prepare ICD-85 loaded sodium alginate NPs and investigate their ability to inhibit proliferation of cancer cells. ICD-85 used in the present study is combination of 3 peptides isolated partially from two different venoms [28]. The combination of these peptides used because they work together synergistically having anti-proliferative activity along with anti-angiogenic activity [39]. The natural polymer alginate was chosen in the present study as the best candidate for drug carrier owing to its biocompatibility, biodegradability, easily obtained at a low cost and non-toxic [4], [7].

The ability of the ionic gelation process to form ICD-85 loaded NPs was assessed by employing FT-IR to determine ICD-85 and sodium alginate interactions. Based on the FT–IR spectra, the strong and broad peaks in the 3421 cm^{-1} ranges correspond to O–H stretching and intermolecular hydrogen bonding. We can see carboxyl peaks near 1642 cm^{-1} (symmetric – COO^- stretching vibration) and 1421 cm^{-1} (asymmetric COO^- stretching vibration).

The DLS analysis revealed that the ICD-85 loaded NPs had a mean hydrodynamic diameter of 300 nm. However, TEM revealed the ICD-85 loaded NPs to have a size of approximately 200 nm. The mean nanoparticle diameter measured using TEM is significantly smaller than the mean diameter obtained with the DLS method. Nanoparticles appeared to be considerably smaller when viewed with TEM as compared to the average particle size observed with DLS [40]. On the other hand DLS measures the apparent size (hydrodynamic radius) of a particle, including hydrodynamic layers that form around hydrophilic particles, leading to an overestimation of nanoparticles size [41]. Hence the discrepancy in the size of nanoparticles is because the DLS method gives the hydrodynamic diameter rather than the actual diameter of hydrophilic nanoparticles [41].

Zeta potential is quite important for colloids and nanoparticles in suspension. Its value is closely related to suspension stability and particle surface morphology [42], [43]. The zeta potential of the sodium alginate NPs was –21.9 mV and changed to –16.1 mV after loading with ICD-85. The negative surface charge of sodium alginate NPs may be attributed to the presence of ionized carboxyl groups of alginate segments on the nanoparticles surface. These results indicate that the loading of ICD-85 decreased the surface potential of ICD-85 loaded NPs. High absolute value of the zeta potential suggests high surface charge of the nanoparticles, which leads to strong repellent interactions among the nanoparticles in dispersion [44]. Thus, this change in the zeta potential of sodium alginate NPs is indicative of successful loading with ICD-85.

Our nanoparticulate formulation solely exhibited a sustained release phenomenon, under in vitro conditions as depicted in figure 3. Alginates offer an inert environment ideal for control drug release at a desirable rate [45]. The main release mechanism in encapsulated drugs from alginate pellets is by diffusional processes through pores and the release is facilitated by the degradation of the polymeric network [45]. Relatively initial burst release should be owed to the presence of free ICD-85 absorbed on the surface of NPs, while the sustained release was attributed to the cleavage of the chemical bond between ICD-85 and sodium alginate particles [32]. Even after 60 h, about 10% of the ICD-85 still remained in the NPs, which were found to suitable sustained release phenomenon of ICD-85 NPs. Our previous study revealed that ICD-85 is stable throughout the 24 hours in the culture medium [28]. ICD-85 loaded NPs had high loading capacity (90.48%) and suitable sustained release profile. These results indicated another advantage of the ICD-85 loaded NPs versus free form of ICD-85, which were found to release the ICD-85 too slowly from NPS form as compared to free ICD-85.

When examined for its ability to suppress the growth of human larynx carcinoma cells, we found that ICD-85 loaded NPs significantly more potent than free ICD-85. Numerous investigations have shown that nanoparticulate drug delivery systems can increase anti-tumor efficacy while reducing side effects [46], [47]. Therapeutic potentiality of the ICD-85 loaded NPs was investigated by MTT based cell proliferation assay. MTT results showed a sharp discrimination in cell inhibition between free ICD-85 and ICD-85 loaded NPs, thus stressing the key role of NPs binding and internalization in enhancement of cytotoxic activity [32]. Our results are in accordance to Zhang *et al*. [48] who demonstrated that the 10-hydroxycamptothecin-loaded NPs developed a higher in vitro cytotoxicity and superior in vivo anti-tumor activity in mice than the free drug. Similar results have been also obtained by Hong *et al*. [49] who indicated that the HCPT loaded NPs exhibited anti-tumor effects enhancement as compared to free HCPT.

In the cell cytotoxicity assay, free NPs did not significantly affect the viability of HEp-2 cells. These results indicated that free NPs itself did not affect the proliferation of tumor cells. Therefore, the interference of the carrier itself was negligible in this study and hence, we can anticipate that ICD-85 loaded NPs show significant cytotoxicity on HEp-2 cell line compared to free form of ICD-85. Our results are in accordance to Ravindran *et al.* [50] who showed that Thymoquinone loaded NPs was able to reduce the IC_{50} value in KBM-5 cancer cells from 3.85 µM for free Thymoquinone to 1.9 µM for Thymoquinone loaded NPs. Similar results have been also obtained by Li *et al.* [51] who indicated that the cisplatin-loaded NPs exhibited a superior anti-tumor effect against cancer cell lines compared to free drug.

5 CONCLUSION

We have developed a natural polymer based nanoparticle delivery system for ICD-85 as cytotoxic agent. ICD-85 was easily loaded to the sodium alginate NPs with high loading capacity and sustained release phenomenon. Considering the negligible interference of sodium alginate NPs on cytotoxicity of HEp-2 cells, we can anticipate that ICD-85 loaded NPs has significant cytotoxicity on HEp-2 cell line compared to free form of ICD-85 which indicate the potential of the nano-encapsulation to improve the cytotoxicity of ICD-85. The simplicity of preparation, the high drug loading capacity and improved efficacy, encourage further evaluation of the nano-formulation in animal model.

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