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Saponin-loaded chitosan nanoparticles and their cytotoxicity to cancer cell lines *in vitro*

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ABSTRACT

In this work we developed a nanoformulation for anticancer saponin with chitosan for an enhanced and sustained release. The saponin loaded chitosan nanoparticles showed a particle size of 65 ± 7 nm. The synthesized nanoparticles were analyzed by FTIR, TG/DTA, SEM and AFM. The cytotoxicity of the nanoparticles was analyzed on L929, NIH-3T3, KB and PC3 which showed particles are non-toxic in a concentration range of 0.1–1.0 mg/ml whereas the nanosaponin showed specific toxicity on PC3 and KB cell lines. The internalization of the nanosaponin on L929 and PC3 was confirmed by Rhodamine conjugation with the nanoparticles. Our preliminary studies support that nanosaponin could be an efficient therapeutic agent for cancer.

1. Introduction

Saponins are naturally occurring amphiphilic compounds present in many foods of plant origin. Legumes, in particular, are rich sources of dietary saponin. Although practically non-toxic to man upon oral ingestion, saponins are powerful hemolytic agents when injected into the bloodstream. Saponins are lipase inhibitors (Basalingappa & Chandravadan, 1971; Fenwick & Oakenfull, 1983; Francis, Kerem, Harinder Makkar, & Becker, 2002; Nair, Kalariya, & Chanda, 2005; Oakenfull & Sihdu, 1990; Oakenfull, 1981; Yoshizumi et al., 2006; Zhao et al., 2005). Populations consuming diets high in legumes and their products are therefore exposed to a highlevel of saponin. There is an increased interest in the importance of different micronutrients as well as of non-nutritive compounds present in vegetables in the prevention of chronic diseases such as cardio vascular heart disease and cancer (Shibata, 2001; Sung, Kendall, & Rao, 1995; Yibing, Yichun, & Biao, 2007). Saponin possesses important biological activities, including hypocholesterolaemic, immune-stimulatory (Potter, 1993; Wu et al., 1990) and antitumorigenic (Yu et al., 1992) effects. However the insolubility of these saponins limits their wide spectrum actions in medical field.

Chitosan is a well known biopolymer having many applications in tissue engineering, wound healing (Muzzarelli, 2009; Sudheesh Kumar et al., 2010), drug delivery (Elzatahry & Mohy, 2008; Kashappa & Hyun, 2005; Kofuji, Ito, Murata, & Kawashima, 2001; Thanou, Verhoef, & Junginger, 2001), and also in gene delivery (Jayakumar, Chennazh, et al, 2010; Muzzarelli, 1988, 2009, 2010; Richardson, Kolbe, and Duncan, 1999). Chitosan nanoparticles used for the delivery of polypeptides such as insulin, tetanus toxoid, and diphtheria toxoids are widely explored (Calvo, Remunan, Vila, & Alonso, 1997a; Calvo, Remunan, Vila, & Alonso, 1997b; Huang, Khor, & Lim, 2004; Janes & Alonso, 2003; Vander et al., 2003; Xu & Du, 2003).

Nano sized drug delivery vehicles formulated from biocompatible chitosan and biodegradable polymers constitute an evolving approach to saponin delivery and tumor targeting (Jayakumar, Deepthy, Manzoor, Nair, & Tamura, 2010). Biodegradable saponin carriers are being purposely engineered and constructed with nanometer dimensions. Such approaches made it possible to develop smart materials like nanosaponin as a better therapeutics. In this paper we are describing about synthesis, characterization and anticancer activities of nanosaponin in detail.

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2. Materials and methods

2.1. Materials

Chitosan (viscosity average molecular weight 20 kDa, degree of *N*-deacetylation (75–80%) was purchased from Koyochitosan Company, Japan and used as received. Tripoly phosphate (TPP) was received from Sigma–Aldrich Company, Bangalore.

2.2. Preparation of plant extract

The plant extract was prepared from *Sapindus emarginatus*. The leaves were washed in tap water, shade dried for 10 days and made into a fine powder of 40 mesh size using the laboratory mill. Following that, 100 g of the powder was filled in the thimble and extracted using 500 ml of distilled ethanol in soxhlet apparatus for 8–10 h. The extract was filtered through Whatman No. 1 filter paper to remove all unextractable matter, including cellular materials and other constitutions that are insoluble in the extraction solvent. The entire extract was concentrated to dryness using rotary flash evaporator under reduced pressure. The dried extract was redissolved in ethanol to yield solutions containing 50, 100, 200 and 300 mg of leaf extract per ml solvent.

2.3. Preparation protocols

2.3.1. Synthesis of chitosan nanoparticles

Chitosan-NPs were obtained by ionic cross-linking of chitosan with TPP, which were described in the literatures (Anitha et al., 2009). The prepared nanoparticles were separated by centrifugation at 20,000 rpm for 1 h, and then purified, dispersed in water and lyophilized for further analysis.

2.3.2. Synthesis of nanosaponin

Nanosaponin was prepared by a simple ionic cross-linking method using TPP through aqueous route. Briefly, saponin (5 mg in 1 ml ethanol) and chitosan (50 mg in 5 ml 1% acetic acid) were stirred for 5 min at 500 rpm. Further, the whole system was mixed with 150 μl TPP solution followed by stirring for 20 min at 500 rpm. The nanoparticle suspension was then centrifuged at 12,000 rpm for about 45 min and the residue was resuspended in milli Q water till the pH became 7.4.

2.3.3. Synthesis of Rhodamine conjugated nanosaponin

Rhodamine (40 μl) with saponin (5 mg in 1 ml ethanol) and polymer (50 mg in 5 ml 1% acetic acid) were stirred for 20 min at 500 rpm. Further the whole system was mixed with 100 μl TPP solution followed by stirring for 20 min at 500 rpm. The resulting solution was lyophilized to get the powder sample. Then it is used for cell uptake studies.

2.4. Analytical determinations

FT-IR spectra of chitosan, chitosan-NPs, saponin and nanosaponin were recorded on Perkin Elmer Spectrum RX1 Fourier transforms infrared spectrophotometer using KBr. NMR analysis of the plant extract has been analyzed to check the chemical components present in the saponin. Both H^1 and ^{13}C NMR analyses were done on the extract. ^1H NMR spectra of the samples were recorded on a Bruker DPX 300 NMR spectrometer using tetramethylsilane as an internal standard and D_2O as solvent at 25 °C. Thermal studies were done by SII TG/DTA 6200 EXSTAR instrument. The thermal stability and thermal decomposition of prepared systems were investigated using TGA are given in Fig. 4. The temperature scale is 10 °C/min. The patterns of pure saponin were obtained using the X-ray diffractometer (PANalytical X'Pert PRO) with Cu source of radiation. Measurements were performed at voltage of 40 kV and 25 mA. The scanned angle was set from $3^\circ \leq 2\theta \geq 40^\circ$ and the scan rate was 2° min^{-1}. The particle size was measured by Dynamic light scattering (DLS-ZP/Particle Sizer Nicomp™ 380 ZLS) taking the average of 3 measurements and zeta potential was estimated on the basis of electrophoretic mobility under an electric field as an average of 30 measurements. The surface morphology of nanoparticle was analyzed by scanning electron microscope (SEM) (JEOLJSM-6490LA) and Atomic force microscopy (AFM) (JEOL JSPM-5200). The nanoparticle suspension was placed on the silicon wafer with the help of micropipette tip and allowed to dry overnight in air. The cantilever used for the scanning 325 μm in length and 26 μm in width with a nominal force constant of 0.1 N/m. Images were obtained by displaying the amplitude signal of the cantiliver in the trace direction, and the height signal in the retrace direction, both signals being simultaneously recorded.

2.5. Entrapment efficiency

The percentage of saponin incorporated during nanoparticles preparation was determined by centrifuging the saponin-loaded nanoparticles at 30,000 rpm for 30 min and separating the supernatant using HERMLE centrifugation. The supernatant was assayed by UV spectrophotometer (UV-1700 Pharma Spec) at 428 nm by dissolving in ethanol. The calculated entrapment efficiency (EE) was 92%.

2.6. In vitro quantification of saponin from nanosaponin

For *in vitro* quantification of saponin, a standard solution of saponin in ethanol was prepared by dissolving 5 mg of saponin in 100 ml ethanol solution. A serial dilution from 0.2 to 2 ml was taken and diluted up to 25 ml and assayed the system at 428 nm. The data plotted to get a straight line for the quantification of unknown saponin in the nanoparticle.

2.7. In vitro saponin release

A known amount of lyophilized (50 mg) encapsulated saponin was dispersed in 10 ml phosphate buffer, pH 7.4, and the solution was divided into 30 epppendorf tubes (500 μl each). The tubes were kept in a thermo stable water bath set at 37 °C temperature. Free saponin is completely insoluble in water; therefore at predetermined time intervals the solution was centrifuged at 5000 rpm for 7 min to separate the released saponin (which will be in a pellet form) from the loaded nanosaponin. The released saponin was redissolved in 3 ml ethanol to assay spectrophotometrically at 428 nm. The concentration of released saponin was then calculated using standard curve of saponin in ethanol. The percentage of saponin released was determined from the following equation:

$$\text{Release (\%)} = \frac{\text{released saponin from nanosaponin}}{\text{total amount of saponin in nanosaponin}} \times 100$$

2.8. Cell culture

PC3 (Prostate cancer cell line, NCCS Pune) was maintained in Dulbecco's modified Eagles Medium (DMEM) supplemented with 10% fetal bovine serum (FBS). L929 (mouse fibroblast cell line, NCCS Pune), NIH-3T3 (Mouse Embryonic fibroblast cell line, NCCS Pune) and KB (Oral Cancer cell line, NCCS Pune) were maintained in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS). The cells were incubated in CO_2 incubator with 5% CO_2. After reaching confluency, the cells were detached from the

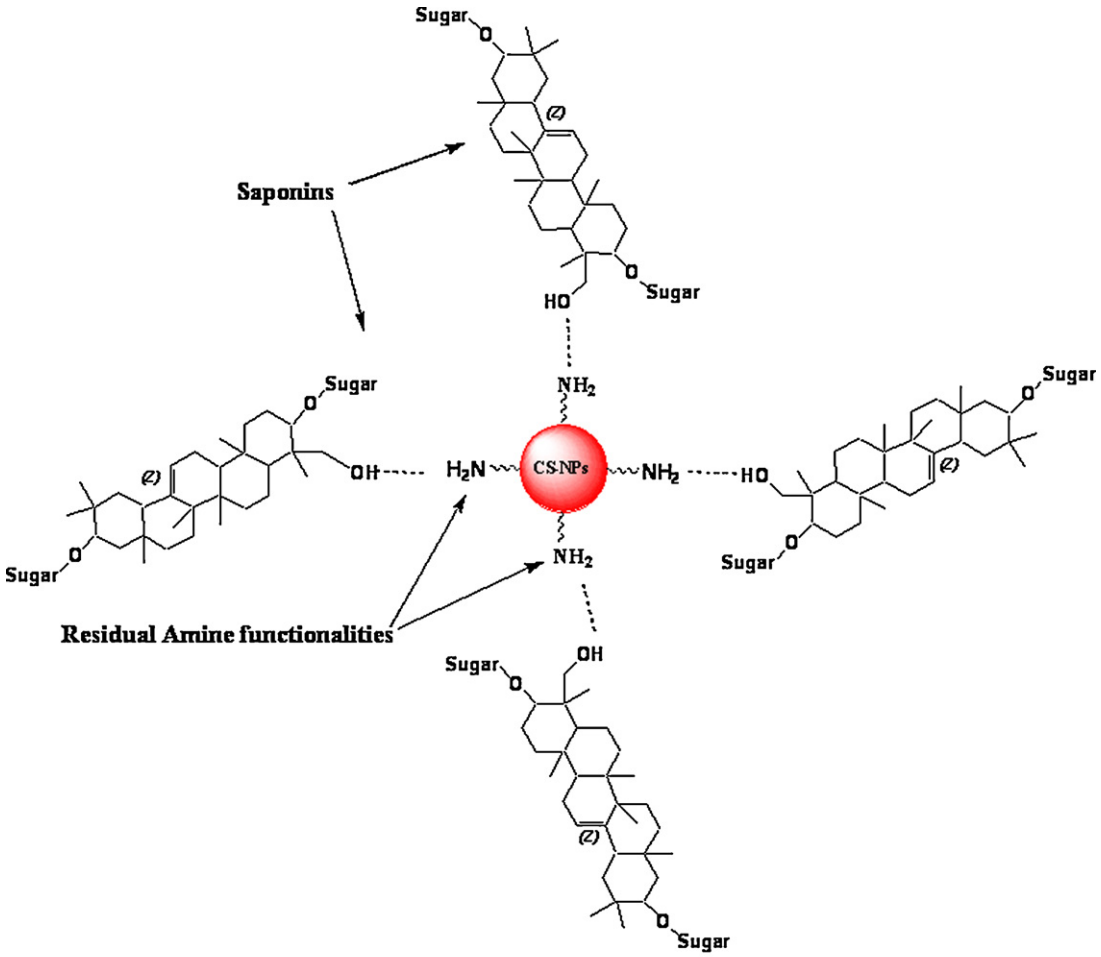

Fig. 1. Hypothesized loading chemistry of saponin in chitosan nanoparticles.

flask with trypsin–EDTA. The cell suspension was centrifuged at 3000 rpm for 3 min and then re-suspended in the growth medium for further studies.

2.9. Cytotoxicity studies

For cytotoxicity experiments, L929, NIH3T3, KB and PC3 cells, respectively were seeded on a 96 well plate with a density of 10,000 cells/cm^2. MTT [3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyl tetrazolium] assay was used to evaluate cytotoxicity of the prepared nanoparticles and this is a colorimetric test based on the selective ability of viable cells to reduce the tetrazolium component of MTT in to purple colored formazan crystals. Six different concentrations of the nanoparticles (0.1, 0.2, 0.4, 0.6, 0.8 and 1 mg/ml) were prepared by dilution with the media. The concentration of saponin in the nanoformulations was varied as follows in five concentrations. (0.1 mg/ml) of nanoformulation has saponin concentration as {[saponin]} = 0.95 μg, likewise (0.2 mg/ml) of nanoformulation contains 1.9 μg, (0.4 mg/ml) {[saponin]} = 3.8 μg, (0.6 mg/ml) {[saponin]} = 4.6 μg, (0.8 mg/ml) {[saponin]} = 7.6 μg and a higher concentration as (1 mg/ml) {[saponin]} = 8.6 μg, respectively. After reaching 90% confluency, the cells were washed with PBS buffer and different concentrations of the nanoparticles (100 μl) were added and incubated. Cells in media alone devoid of nanoparticles acted as negative control and wells treated with Triton X-100 as positive control for a period of 24 h. 5 mg of MTT (Sigma) was dissolved in 1 ml of PBS and filter sterilized. 10 μl of the MTT solution was further diluted to 100 μl with 90 μl of serum-free phenol red free medium. The cells were incubated with 100 μl of the above solution for 4 h to form formazan crystals by mitochondrial dehydrogenases. 100 μl of the solubilisation solution (10% Triton X-100, 0.1 N HCl and isopropanol) was added in each well and incubated at room temperature for 1 h to dissolve the formazan crystals. The optical density of the solution was measured at a wavelength of 570 nm using a Beckmann Coulter Elisa plate reader (BioTek Power Wave XS). Triplicate samples were analyzed for each experiment.

2.10. Cellular uptake studies

Acid etched cover slips kept in 24 well plates were loaded with L929 and PC3 cells with a seeding density of 5000 cells per cover slip and incubated for 24 h for the cells to attach well. After the 24 h incubation the media were removed and the wells were carefully washed with PBS buffer. Then the particle at a concentration of 1 mg/ml was added along with the media in triplicate to the wells and incubation for 4 h. Thereafter the media with sample were removed and the cover slips with well attached cells were processed for fluorescent microscopy. The processing involved washing the cover slips with PBS and thereafter fixing the cells in 3.7% para formaldehyde (PFA) followed by a final PBS wash. The cover slips were air dried and mounted on to glass slides with DPX as the mountant medium. The slides were then viewed under the fluorescent microscope.

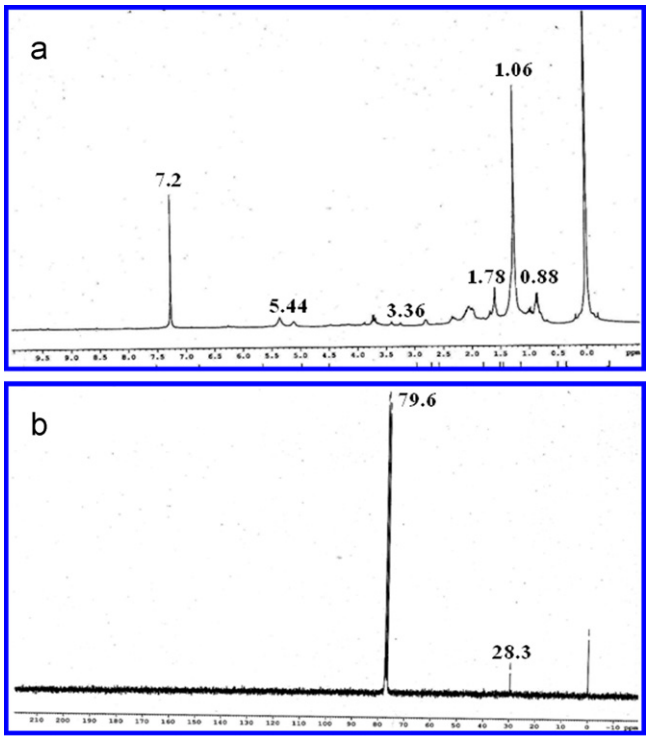

Fig. 2. (A) ^{1}H NMR and (B) ^{13}C NMR spectrum of saponin extract.

2.11. Statistics

Statistical analysis of the data was performed via one-way analysis of variance (ANOVA) using origin software; a value of $p < 0.05$ was considered significant ($n = 3$).

3. Results and discussion

3.1. Preparation of extract

The plant extract was prepared from *S. emarginatus* and the presence of saponin was confirmed through NMR and FTIR.

3.2. Nuclear magnetic resonance spectroscopic analysis of plant extract

The H^{1} NMR and ^{13}C NMR analysis of the plant extract analyzed to check the chemical structure of the saponins.

3.2.1. $_{1}H^{1}$ NMR

From Fig. 2A, the singlet peak at 7.2 (ppm) represents the presence of aromatic functional groups. The methylene protons are identified at 0.88 ppm and the sugar methylene protons were identified at 1.06 ppm (Jangwan, Dhobhal, & Naveen, 2010; Han et al., 1999). The H-3, H-12 and H-18 protons in the saponin have been identified at sharp peaks at 3.36, 5.44 and 3.14 ppm, respectively.

3.2.2. ^{13}C NMR spectra of plant extract

^{13}C NMR spectra of the saponin analysis done in order to confirm the presence of functional groups such as trisaccharides, carboxylic acids, aliphatic acids, esters and amides, saponin, glycosides. Fig. 2B represents the ^{13}C NMR spectra of the extract, saponin, which confirmed the above said functionalities in the extract. The sugar moieties have been identified at 79.6 ppm with a sharp peak and the carbon skeleton has been determined as it showed a prominent peak at 28.3 ppm, which is characteristic for the C-23 skeleton of the saponin. The FT-IR analysis also confirmed the presence of aromatic rings in the saponin molecule (Kizu & Tomimori, 1982).

3.3. Synthesis of nanosaponin

The saponin loaded chitosan nanoparticles were prepared by TPP cross-linking. The nanoparticles are dried and used for further characterization studies. The loading chemistry is shown in Fig. 1. The expected loading chemistry is hydrogen bonding interaction between the saponin and chitosan nanoparticles. Chitosan is having active $-NH_2$ groups which can easily react with the –OH groups of the saponin. Moreover, the sugar moieties in the saponins could be utilized for the weak interactions with carrier chitosan molecules. The hydrogen bonding is enough to hold saponin on the chitosan nanoparticles. The new peak at 1560 cm^{-1} indicates there is a possibility for amide linking between saponin and chitosan. Since the $-NHCOCH_3$ in chitosan is very less, there would not be any hydrogen bonding interaction between –OH and $-NHCOCH_3$ groups.

3.4. Particle size and topography

The particle size analysis done with dynamic light scattering was presented below (Fig. 3), bare chitosan-NPs showed particles size in the range of 35 ± 7 nm whereas the nano saponin showed increased size of 55 ± 7 nm. The surface morphology of nano saponin was shown in Fig. 3D. The SEM and AFM analyses give a good evidence for the entrapment of the saponin on the chitosan nanoparticles sine the nano saponin has higher particle size.

3.5. FTIR studies

Chitosan-NPs were prepared by ionic gelation technique using TPP as cross-linker in aqueous medium. To confirm the nanoformulation of chitosan, FTIR analysis was done, characteristic peaks of bare chitosan are located at 1644, 980 and 3436 cm^{-1} which corresponds to amide I, anhydro glucosidic ring (Fig. 4A) and primary amine, respectively. These observed peaks get shifted from higher wave number region to lower wave number region as 1644–1639 cm^{-1}. The reduction in stretching frequency could be attributed to the TPP interaction with the amine functionality; thereby the bond length of amine would be disturbed or increased. As the bond length increased, the stretching frequency would be decreased, thereby wave number shifts from higher frequency regions to lower frequency region (Sanoj Rejinold, Muthunarayanan, et al., 2010; Sanoj Rejinold, Chennazhi, Nair, Tamura, & Jayakumar, R, 2011). The saponin loading was confirmed by the presence of a new peak at 1560 cm^{-1} corresponds to amide linkage between saponin and chitosan nanoparticles. There was a sharp peak at 3450 cm^{-1} region which assures that interaction of saponin with chitosan is more of hydrogen bonding than ionic interactions.

3.6. Thermal analysis

The thermal stability and thermal decomposition of prepared systems were investigated by TG and are given in Fig. 4B. It shows that the initial degradation temperature of chitosan is very close to 280 °C, slow weight loss starting from 140 to 200 °C due to the decomposition of polymer with low molecular weight, followed by more obvious loss of weight starting from 200 to 310 °C, which could be attributed to a complex process including dehydration of the anhydro glucosidic ring (Devika &Varsha, 2006; Radhakumary, Nair, Suresh, & Nair, 2005). The degradation profile of chitosan-NPs seems to be different compared to chitosan, but stabler than chitosan which proves that the system is of amorphous nature. The

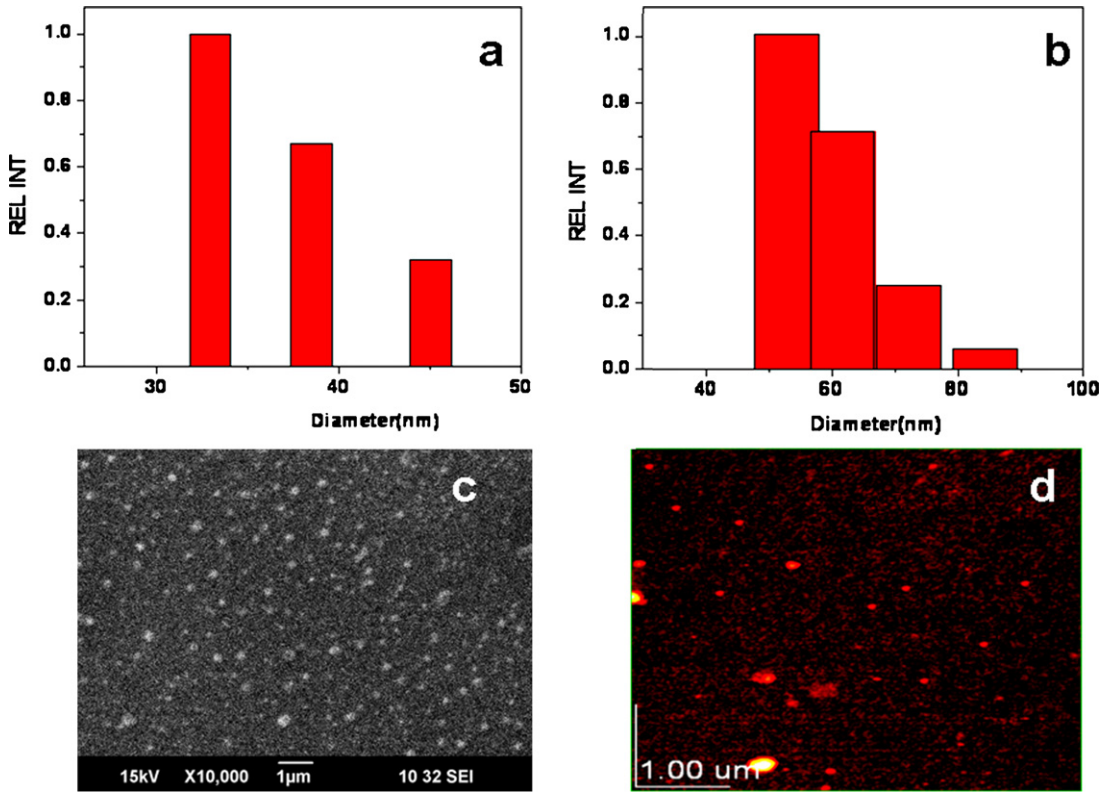

Fig. 3. DLS spectra of (A) chitosan-NPs; (B) nanosaponin; (C) SEM of nanosaponin and (D) AFM of nanosaponin.

saponin alone has lower stability compared to the chitosan (control) and chitosan-NPs that show comparatively higher stability. The saponin alone showed a sharp degradation at 100 °C which indicates the melting point is at this temperature range. However the nanosaponin has greater stability even at 500 °C and 34% remnant at the same temperature.

Similarly, the differential thermal analysis of all the systems was preformed to understand the behavior of the chitosan, chitosan-NPs, saponin and nanosaponin on application of thermal energy. Polysaccharides usually have a strong affinity for water and in solid state these macromolecules may have disordered structures that can be easily hydrated. The hydration properties of polysaccharides depend on primary and supramolecular structures. The endothermic peak related to evaporation of water is expected to reflect the molecular changes brought in after cross-linking. Thus, chitosan and modified systems had different varied water-holding capacity. In chitosan, the bound water molecules are associated with hydrophilic hydroxyl groups. The thermogram of chitosan showed endothermic peak at 95 °C. The saponin alone showed a sharp endothermic peak at 100 °C which is shifted to higher temperature region in the nanosaponin.

The heat capacity of chitosan-NPs was found to be less compared with that of chitosan. The cross-linking reaction via TPP modifies the crystalline nature of chitosan and also the nanosaponin. The DTA analysis showed saponin has started to melt at 100 °C wherein the loaded nanosaponin, the same endothermic peak was shifted to 350 °C which assures the amorphous nature of the nanosaponin (Fig. 4C).

On the basis of these results it can be stated that increase in the polar groups and reduction in crystalline domains caused reduction in heat capacity/thermal stability. The second thermal event observed was the presence of exotherms due to the decomposition of the polymer. Owing to the differences in the chemical characteristics, changes in the exothermic peak of chitosan and cross-linked chitosan were also observed. Characterization of cross-linked chitosan polymer and its saponin loaded nanoparticles were analyzed by FTIR, which provided the evidence of reduction in crystallinity after cross-linking with TPP. The whole TG/DTA analysis confirms the more amorphous nature for the cross-linked nanosaponin.

3.7. Nature of saponin in chitosan nanoparticles (XRD studies)

The characteristic peaks of saponin exhibited as shown in Fig. 5 and can be inferred to traits of a high crystalline structure. The characteristic peak of saponin $2\theta = 16^{\circ}$ (Fig. 5A) was observed but the intensity was less when entrapped into nanoparticles (Fig. 5B), possibly due to formation of an amorphous complex with the intermolecular interaction occurring within the matrix. A similar phenomenon has been observed in the literature providing evidence that the crystalline structure of saponins was converted to an amorphous state (Abdelwahed, Degobert, Stainmesse, & Fessi, 2006).

3.8. In vitro saponin release

The saponin EE of chitosan-NPs was found to be 95%. The *in vitro* saponin release was studied with PBS at pH 7.4. The percentage of saponin released from chitosan-NPs at predetermined time intervals was calculated using the standard curve prepared for saponin. Fig. 5C shows the *in vitro* saponin release profile of saponin. Release occurs by a combination of diffusion of the saponin out of the particles into the external environment and also by the degradation of the polymer. The decrease of the saponin release at longer time suggested the importance of the diffusion process in the release kinetics (Dev, Jithin, Nair, & Jayakumar, 2010). The *in vitro* drug release profile shows that 32% of saponin was released within 12 h. After the initial burst, a slow release was observed. A sustained and controlled release has been observed till 72 h and 75% of the

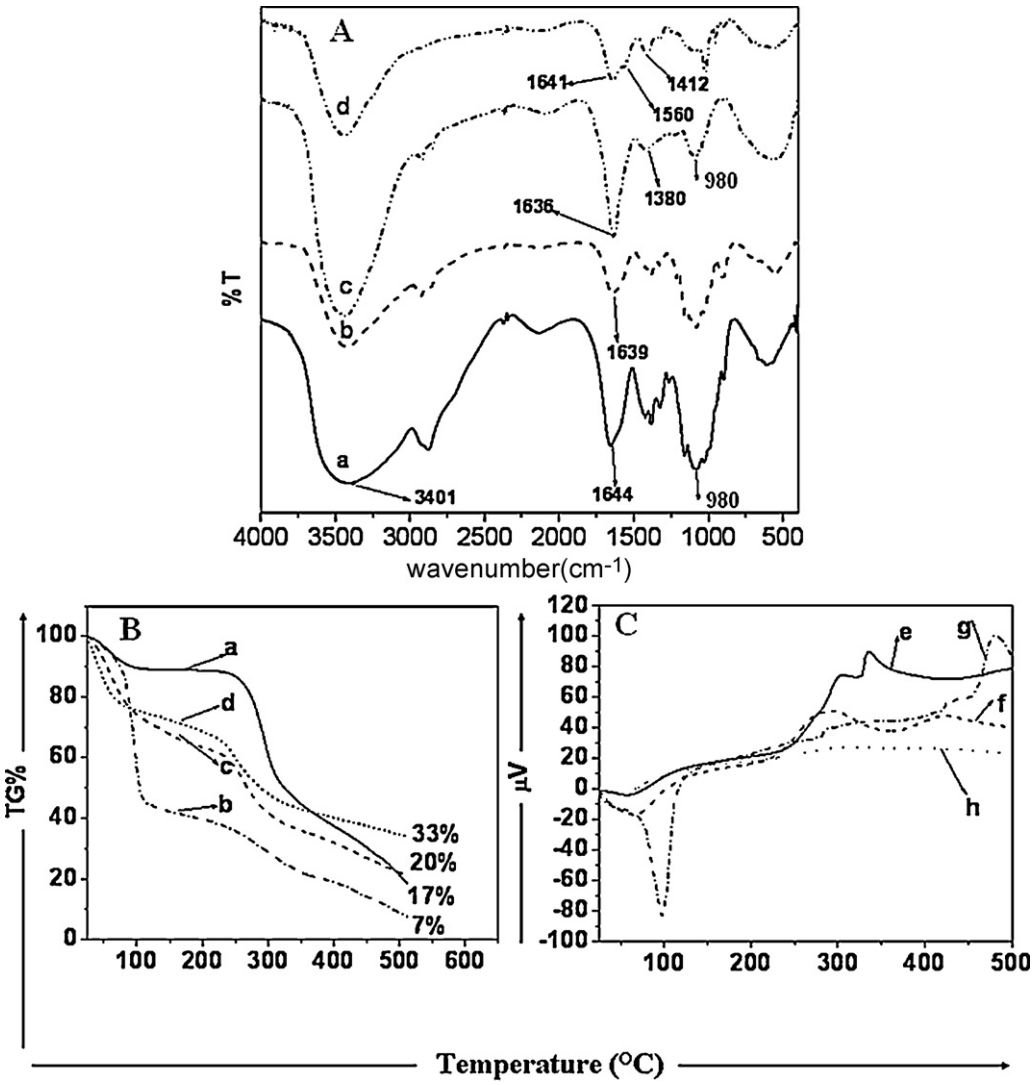

Fig. 4. (A)FT-IR spectra of (a) chitosan (control), (b) chitosan-NPs, (c) saponin, and (d) nanosaponin. Thermal analysis: (B) TG and (C) DTA profile for (a) chitosan (control), (b) chitosan nanoparticles, (c) saponin and (d) nanosaponin.

loaded saponin released during this period. The initial burst release is expected due to the saponin molecules attached on the surface of the chitosan-NPs, and the sustained release is from the entrapped saponin. The observed sustained release after initial burst was significant because controlled release is required in the field of cancer therapy. These results indicated that the nanosaponin is useful controlled delivery system for cancer treatment.

3.9. Cytotoxicity studies

The synthesized chitosan-NPs were analyzed by MTT for its toxicity on normal as well as cancer cells. As determined by MTT assay, the chitosan-NPs were non-toxic on L929, NIH-3T3, PC3 and KB cells (Fig. 6A). The nanosaponin showed specific toxicity on prostate and oral cancer cells (Fig. 6B) while it did not show any toxicity on normal L929, and NIH-3T3 cells (Fig. 6B). The cytotoxicity difference between normal and cancer cells by nanosaponin however is unknown, whereas the saponins specific action on cancer cells has been well explored.

Saponins isolated from different plants and animals have been shown to specifically inhibit the growth of cancer cells *in vitro* (Konoshima et al., 1998; Kuznetzova, Anisimov, & Popov, 1982; Marino, Iorizzi, Palagiano, Zollo, & Roussakis, 1998; Mimaki, Kuroda, Kameyama, Yokosuka, & Sashida, 1998; Podolak, Elas, & Cieszka, 1998; Rao & Sung, 1995). The pursuit of natural substances capable of controlling malignancies has led to considerable research on this property of saponins. The previous research work on isolated saponin from *Gymnema sylvestre* leaves have required 50 μg/ml to show toxicity on Hela cells (human cervical carcinoma) where as it was non toxic to Vero cells (Khanna & Kannabiran, 2009). Similarly saponins isolated from *Paris polyphylla* var. *Yunnanensis* have a good anti tumor action (Yan, Zhang, Gao, Man, & Wang, 2009). It throws light on the fact that there is some molecular level mechanism for saponin to show specific toxicity on cancer cells. Further studies on this aspect are required emergently for the better understanding of the specific anticancer action of saponin.

3.10. Cell uptake studies

Systematic study for cellular uptake of Rhodamine 123 conjugated nanosaponin by L929 cells and PC3 was performed by visualizing the fluorescence of Rhodamine 123 using fluorescent microscopy. Rhodamine 123 is a green fluorescent dye, so the cells with nanoparticle uptake would typically appear bright green. Fluorescent microscopic images taken after 24 h of incubation revealed that, there was significant internalization and retention of nanopar-

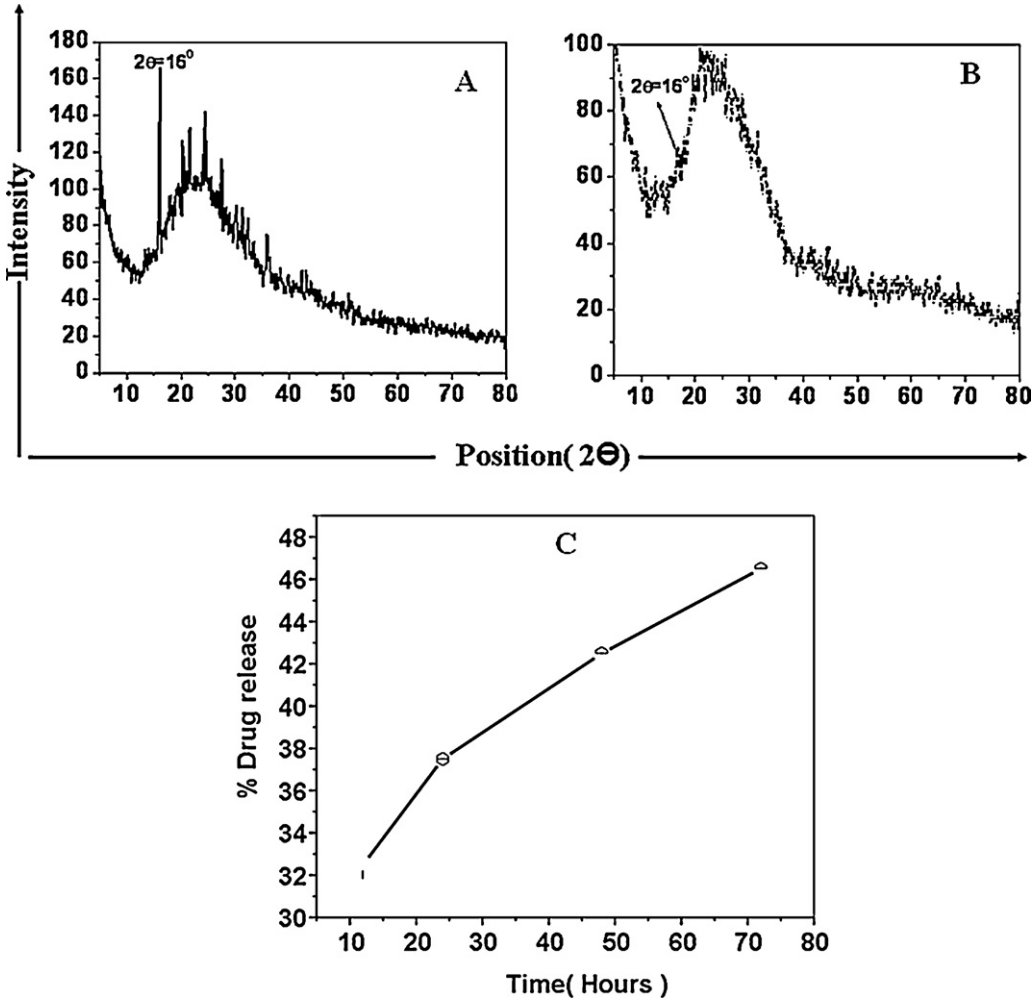

Fig. 5. XRD spectra of (A) saponin, (B) nanosaponin, and (C) *in vitro* release of saponin from nanosaponin (values reported are mean ± SD; $n = 3$).

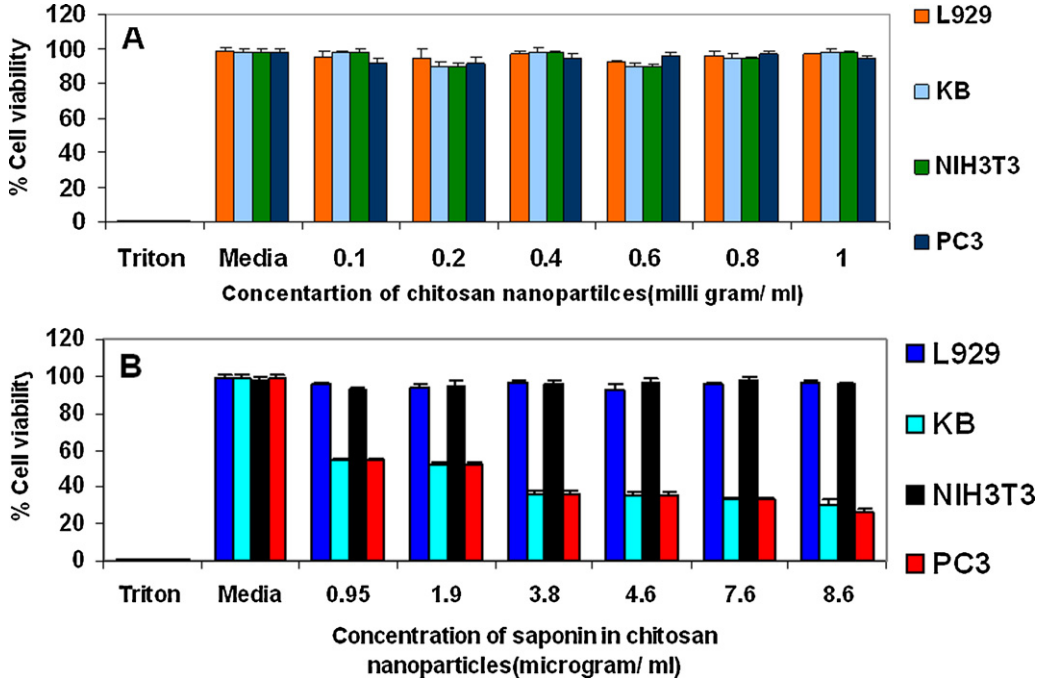

Fig. 6. (A) MTT assay of chitosan-NPs and (B) nanosaponin on L929, KB, NIH3T3 and PC3 cells (values reported are mean ± SD; $n = 3$).

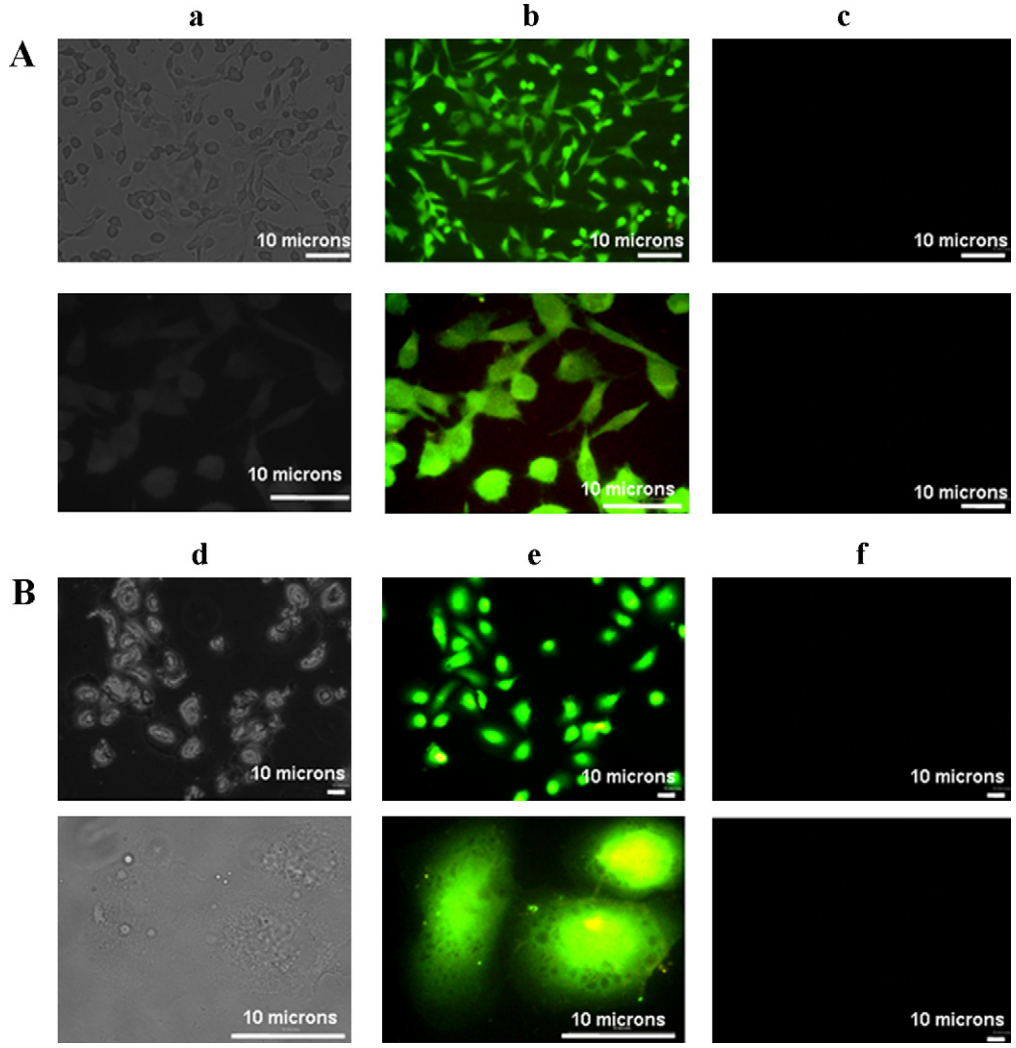

Fig. 7. Fluorescent microscopic images of nanosaponin on (A) L929 and (B) PC3: (a & d) bright field, (b & f) fluorescent images, and (c & e) control cells at different magnifications.

ticles inside the cells (Fig. 7A and B). Images of control cells without any particles did not show fluorescence, which further validates the study. Additional experiments are underway to give more insight to cellular uptake and sub-cellular localization of nanosaponin.

4. Discussion

In recent years considerable attention has been given to advanced biomaterials field including nanobiomaterials, smart hydrogels, etc. This study presents the preparation and evaluation of saponin loaded chitosan nanoparticles (nanosaponin) as a cancer therapeutic agent. The methodology is simple and less time consuming.

Saponin isolated from different plants are well known for their potential anticancer activity, however, the reported value for saponin is 50 μg/ml to show toxicity against cancer cells.

The anticancer action of saponins on cancer cells may take place through diverse and complex mechanisms. The destructive activity of saponins against cells such as erythrocytes was, however, not related to cytostatic activity against cancer cells (Mimaki et al., 1998). The selective inhibition of the growth of tumor has been observed by triterpenoid saponins (avicins from Acacia victoriae) by cell cycle arrest in human breast cancer cell line and apoptosis in leukaemia cell line (Mujoo et al., 2001) which in turn reduced both tumor incidence and multiplicity in a murine skin carcinogenesis model (Hanausek et al., 2001). Saponin (triterpenoidor steroid)-induced apoptosis is primarily caused by stimulating the cytochrome c–caspase 9–caspase 3 pathway in the human cancer and other cell lines (Cai, Liu, Wang, & Ju, 2002; Haridas, Arntzen, & Gutterman, 2001; Liu, Xu, & Che, 2000; Yui et al., 2001), a property that is shared by a ginseng saponin derivative (20-O-(b-D-glucopyranosyl)-20(S)-protopanaxadiol) produced in intestine by bacteria and absorbed into blood (Lee, Ko, et al., 2000; Lee, Sohn, Park, Kim, and Jung, 2000). The specific toxic activity against macrophage colony-stimulating factor-induced growth of macrophages by terpenoids (securiosides) having specific structural features such as presence of dimethoxycinnamoyl group has been detected by Yui et al. (2001). Depending on the structural functionalities saponins can induce a cell cycle arrest mediated by inhibition of the phosphatidylinositol-3-kinase–protein kinase signalling pathway (Mujoo et al., 2001) or direct suppression of protein kinase complex genes (Liu et al., 2000) is stimulated by saponins either along with the apoptotic pathway (Mujoo et al., 2001) or independently (Oh & Sung, 2001). The inhibition of the phosphatidylinositol-3-kinase–protein kinase pathway is considered important in apoptosis, given the role of protein kinases in inactivating the caspases (apoptotic enzymes). Saponins also reduce occurrence of reactive oxygen species such as H_2O_2 (Haridas et al., 2001; Pawar, Gopalakrishnan, & Bhutani, 2001), probably by enhancing its breakdown by activation of peroxire-

doxins and catalase, and/or glutathioneperoxidase (Deng & Zhang, 1991) as well as by suppressing its production by inhibiting the phosphatidylinositol-3-kinase signalling pathway (Haridas et al., 2001).

Our study clearly demonstrated that nanosaponin can induce dose-dependent cancer cell death with lower toxicity on normal cells. Moreover, only less than 10 μg is required to show toxicity against cancer cells like PC3 and KB in our study. This is an important observation because cancer cell death has been considered a target of chemotherapeutic agents in a variety of cancer research.

Apoptosis is a process of an essential tissue homeostasis and thus being regarded as the ideal way to inhibit cancer cell growth. Our data is focusing about the anticancer activity of nanosaponin (Fig. 6) and drug release (Fig. 5) which is clearly showing that a sustained release could be possible to cancer cell lines. The bare saponin would completely expire after causing sudden death of a definite population of cancer cell as seen in the previous studies. Nanosaponin would release the drug in controlled fashion to cause toxicity to that population of cells seeded for an MTT assay as well as still hold a potentially toxic quantity of drugs that would have come to use had the population of cells been higher.

Therefore, nanosaponin inducing cancer cell death can be viewed as a new potential generation of cancer treatment. Many studies demonstrated that the induction of cell death in cancer cells by anticancer saponins appeared in a dose and time-dependent manner. Our data is in agreement with this fact showing that the nanosaponin with higher concentrations of saponin can induce cancer cell death within a short-time period of 24 h. Additional studies are required to clarify the efficacy and the mechanism by which the nanosaponin induce apoptotic cell death.

5. Conclusions

In conclusion, the extract from *S. emarginatus* was taken and preliminary phytochemical analysis of the leaf extract was done. The saponin loaded chitosan nanoparticles "nanosaponin", as never reported before, were prepared via a simple cross-linking reaction with TPP for enhanced, controlled and sustained delivery to cancer cell lines. The DLS, SEM and AFM studies confirmed the size of the prepared nanoparticles to be 40–60 nm, which means even after the saponin incorporation, the particle size can be tuned within the optimal range for saponin delivery applications. The thermal studies indicated that nanosaponin has more thermal stability than saponin alone. FT-IR studies confirmed the potential interaction of the saponin with the chitosan nanoparticles. The cellular uptake studies of nanosaponin using L929 and PC3 cells demonstrated significant internalization and retention of nanoparticles inside the cells, suggesting that these nanoparticle systems can be used for delivering saponin directly into the cells. The nanosaponin has showed specific toxicity on cancer cells while they are non-toxic to normal cells. These preliminary results could serve as a good platform for future experimentations with nanosaponin on appropriate experimental animal models with relevance to different human cancers. Our preliminary study thus provide convincing evidence of nanotechnology based saponin delivery to cancer cell lines *in vitro* to enhance the therapeutic efficacy of this well known green chemotherapeutic agent.

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