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Naringin Loaded Chitosan Nanoparticle for Bone Regeneration: A Preliminary *in vitro* Study

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Abstract

Naringin a flavanone glycoside is a major flavanoid in grapefruit. Administration of flavanoids limits their bioavailability due to hydrophobicity and hence nano drug delivery therapy can be employed. To avoid drug associated difficulties, natural polymers are optimal for the preparation of nanoparticle due to their biodegradable nature. Chitosan is the second most abundant biopolymer on earth after cellulose. Chitosan at three different concentrations (0.5%, 1%, 1.5%, 2%) was prepared by ion gelation technique, and one particular concentration was chosen to prepare Naringin loaded chitosan nanoparticle (NCN). The NCN were checked for anti-inflammatory, anti-coagulant, anti-oxidant, cytotoxicity and anti-cancerous activity. The NCN promoted the osteoblast differentiation, it was determined by performing Alkaline Phosphatase (ALP) assay, Picrosirius red staining and alizarin red staining. The results suggest the potential for prolonged and sustained release of Naringin to promote bone formation thereby can be used for bone tissue engineering.

Keywords: Naringin; Nanoemulsions; Nanoparticles

Introduction

The antioxidant properties of flavanoids are exploited in cancer therapy, as it also enhances osteogenesis and inhibits osteoclastogenesis. Osteoporosis is a condition characterized by low bone mineral density (BMD) and micro-architectural deterioration of bone tissue is the characteristic features of osteoporosis which results in an increased risk of fracture [1]. In osteoporosis patients suffering from vertebral and bone fractures show increased risks of morbidity and mortality [2]. The major treatment for prevention and treating osteoporosis is Oestrogen replacement therapy (ERT), it was recently found to be associated with an increased risk in developing endometrial and breast cancer in postmenopausal women [3]. Use of natural food ingredients is in the current trend. Naringin is a citrus flavonoid commonly found in the pericarp (albedo, membrane and pith) of citrus fruits. Recently, the health benefits of naringin on bone have also received much attention. Naringin was shown to improve bone quality in rats with osteoporosis induced by retinoic acid [4] which confirms that naringin is the active ingredient that favours for the bone protective effects of citrus fruit. Naringin significantly increased alkaline phosphatase (ALP) activity, proliferation, and total protein content in rat osteoblastic-like UMR-106 cells [5]. Naringin is highly crystalline in nature, its solubility and bioavailability decreases which limit its clinical role. To overcome the limitation of hydrophobic drugs, non-drug delivery system such as nanoparticles, nanomicelles, nanosuspensions and nanoemulsions can be used. For the preparation of nanoparticle natural polymers can be used determine their bioavailability. Chitosan is a cationic polysaccharide, which has good mechanic property, biocompatibility, antibacterial activity and its nontoxic. Chitosan serves as a promising permeation enhancer and mucoadhesive. Drugs can be encapsulated using chitosan along with Sodium-Tripoly phosphate (TPP) which acts as a cross linker [6].

Materials and Methods

Preparation of naringin from grapefruit rind

The grapefruit was procured from fruit basket. The grapefruit rind was removed with the help of a cork borer. 40.0 grams of the rind was ground with 100 ml of ethanol in a Warring Blender. It was then filtered where the residue and the filtrate were separated into two equal portions. To remove the alcohol, the residue was air dried. One portion of the air dried residue was placed in the extraction thimble; one portion of the filtrate was placed in the extraction flask, which was made up to 250 ml with ethanol. The extraction process was carried out for 5 hours using Soxhlet apparatus.

Preparation of chitosan from shrimp shells

Shrimp was collected from the local market, its head and skin were separated. The shrimp shells were washed with tap water. It was then air dried for a week until the shells became rigid. Isolation of chitosan requires the following three steps demineralisation, deproteinationization of shells and deacylation of chitin [7].

Preparation of chitosan nanoparticle

The Chitosan nanoparticles were synthesised by ionic gelation technique. Chitosan of various concentrations (0.5%, 1%, 1.5%, 2%) were selected, synthesised and characterised by Scanning Electron Microscope (SEM), to standardise the appropriate nanoparticle size. The ratio of Chitosan and TPP was maintained (C: TPP=4:1), as various combinations of Chitosan and TPP were prepared.

Preparation of naringin loaded chitosan nanoparticle (NCNs)

The NCNs were synthesised by ionic gelation technique. By performing SEM analysis the appropriate nanoparticle size was found to be achieved in the Chitosan-TPP concentration (0.5-0.12%). 0.5% of Chitosan (w/v) was dissolved in 1% (v/v) acetic acid. Naringin (100 µM) was added drop wise to the chitosan solution with continuous stirring. The TPP solution which was maintained at 4°C was added

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drop wise to the chitosan solution, under continuous magnetic stirring, for 2 hour [7].

Anti-inflammatory activity

Heat induced haemolysis: The percentage inhibition of the anti-inflammatory activity of the drug was determined and calculated by the below mentioned formula [6].

$$\% \text{ INHIBITION} = 1 - \frac{OD_2 - OD_1}{OD_3 - OD_1} \times 100$$

Inhibition of albumin denaturation: The percentage inhibition of the albumin denaturation was calculated as follows [6].

$$\% \text{ INHIBITION} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \text{ x } 100$$

Anti-coagulant activity: The Prothrombin Time (PT) was determined; plasma sample was divided into three groups:

GROUP I: Negative Control group.

0.2 ml of plasma+0.1 ml of 0.9% saline+0.3 ml of 25 ml $CaCl_2$

GROUP II: Positive Control group.

0.2 ml plasma+0.1 ml of 50 mg/ml EDTA+0.3 ml $CaCl_2$

GROUP III: Test Sample.

0.2 ml plasma+200 µl naringin loaded chitosan nanoparticle+0.3 ml $CaCl_2$.

All three test tubes were incubated at room temperature for 15 minutes. The tubes were tilted at an angle of 45° for every 30 seconds to measure the clotting time. This time is referred to as Prothrombin Time (PT) [7].

***In vitro* antioxidant activity:** To evaluate the antioxidant property DPPH radical scavenging method was used [8].

The percentage inhibition of DPPH free radical was calculated by,

$$\% \text{ INHIBITION} = \frac{Abs_{control} - Abs_{sample}}{Abs_{control}} \text{ x } 100$$

Where,

$A_{control}$=Absorbance of the control (Ascorbicacid)

A_{test}=Absorbance of the reaction mixture sample.

***In vitro* assay for cytotoxicity activity:** The %cell viability of Vero cells was calculated by the below mentioned formula [9].

%Cell Viability=A570 of treated cells/A570 of control cells × 100

***In vitro* anti-cancer activity:** HeLa cells and A549 Lung cancer cells were used for anticancer activity studies. The %cell viability was calculated by the below mentioned formula.

%Cell Viability=A570 of treated cells/A570 of control cells × 100

Collection of cells: The Mouse mesenchymal stem cell line was obtained from *National Centre for Cell Science*, Pune (*NCCS*). The obtained cell lines were further cultured in a T25 flask with DMEM (Dulbecco's Modified Eagles Medium). The cell obtained were trypsinized and counted. The %viability of cell suspension was calculated.

Characterization assays

MTT assay for cytotoxicity analysis: The MTT (3(4,5-dimethlythiazol-eyl) 2,5-diphenly-tetrazoliumbromide assay was performed to evaluate the cell viability.

Alkaline phosphatase assay: The wells seeded with 10^4 cells were then washed with phosphate-buffered saline and alkaline phosphatase activity was assayed on days 7 and 10 using 5-bromo-4-chloro-3-Indolyl Phosphate/Nitro Blue Tetrazolium (BCIP-NBT, Sigma-Aldrich). Absorbance was measured using the UV-Spectrophotometer at 595 nm [10-14].

Picrosirius red staining for collagen deposition: The picrosirius red staining was performed to visualise the collagen deposition in the cells. It was assayed on days 7 to 10. The collagen deposited cells were then visualised and documented using an inverted microscope.

Alizarin red staining for calcium precipitation: The alizarin red s staining was performed to visualise the calcium deposition in the cells. The pictures of the cells with calcium deposition were then documented using inverted microscope [8,15].

Results and Discussion

HPLC for extracted naringin

HPLC chromatography obtained from DMSO fraction shows that many peaks were detected at 285 nm. The Retention Time (RT) of the test sample naringin was 39.47 min, whereas the Retention Time for the standard naringin was 39.51 min (Figure 1). When the HPLC chromatograms of orange and blond grapefruit were done, it resulted in a main peak showing the presence of hesperidin, which is a dominant phenolic compound in oranges. The Retention time (RT) was found to be 33.23 minutes [12].

Sem Analysis of chitosan nanoparticle (CN) and naringin loaded chitosan nanopartile (NCN)

The four different concentration of chitosan nanoparticle prepared, were studied using SEM, to determine their morphology and average particle size and morphology. On performing SEM analysis for all the four different concentration of chitosan nanoparticle it was standardised and concluded that for the chitosan nanoparticle concentration 0.5% Chitosan+0.12% TPP, the particle size was below 100 nm that is 88 nm. Hence that was chosen to incorporate the drug (naringin). 100 µM naringin was incorporated into the standardised chitosan nanoparticle, which sized 88 nm initially now increased to 93 nm on incorporation of the drug. The spherical morphology confirms the encapsulation, morphology and size of the naringin loaded chitosan nanoparticle. When 0.5% chitosan+0.25% TPP nanoparticle was synthesised and analysed for SEM, it resulted in the nanoparticle for which the size was 250 nm (Figure 2) [9].

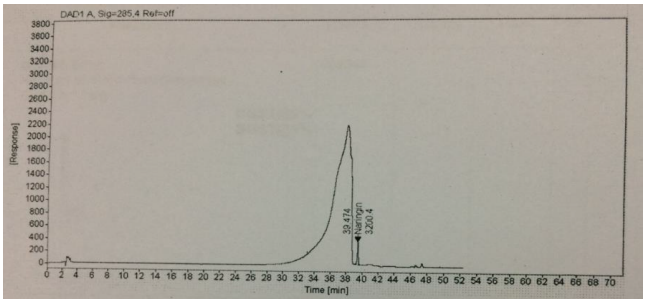

Figure 1: HPLC chromatogram obtained from the dimethyl sulfoxide (DMSO) fraction of grapefruit rind. The main peak is that of naringin, which is the dominant phenolic compound in grapefruit, RT=39.47.

FTIR and XRD Analysis

FTIR analysis was carried out to investigate the chemical interactions between Naringin, Chitosan and TPP, and to identify its functional groups (Figure 3).

The functional group of naringin is OH. The FTIR spectrum of naringin showed the characteristic peak at 1642.09 cm^{-1}. The functional group of chitosan is OH. In the chitosan spectra the strong and wide peak in the range 3500-3200 attributed to hydrogen bonded O-H stretching vibration. The peaks of N-H stretching from the primary amine and type II amide overlapped in this region. The peak for an asymmetric stretch of C-O-C was found at 1072.23 cm^{-1}. The C-N stretching vibration of type I amine was showed in the peak 1319.07 cm^{-1}. The FTIR spectrum of pure Sodium tripolyphosphate (TPP) showed the characteristic bands at P-O stretching 1215.9 cm^{-1}, symmetric and asymmetric vibration of the PO_2 group's 1155.15 cm^{-1}. The FTIR spectrum of Chitosan nanoparticle confirmed that the ammonium group of chitosan is linked to the tripolyphosphorous groups of TPP in the chitosan nanoparticle. In chitosan nanoparticle the tip of the peak of 3464.49 cm^{-1} had a shift to 3339.14 cm^{-1}, which indicates the augmentation of hydrogen bonding. The peaks for the N-H bending were seen at 1654.62 cm^{-1}. In TPP the N-H bending occurs at 1656.55 cm^{-1}, which is shifted to 1654.62 cm^{-1} in chitosan nanoparticle. The cross linked chitosan showed a P=O peak at 1085.73 cm^{-1}, which confirms the formation of chitosan nanoparticle (CN). The above mentioned results indicates the linkage between phosphoric and ammonium ions. In the FTIR spectrum of the naringin loaded chitosan nanoparticle (NCN), characteristic peak of naringin and chitosan were observed. There was a shift in the peak from 1642.09 cm^{-1} to 1655.55 cm^{-1}. It indicates the incorporation of naringin into the chitosan nanoparticle (Figure 4).

The X-ray diffractogram of Naringin showed its crystalline nature, due to intense peak formation. Naringin showed sharp and intense peak at 14°, 14.8°, 18°, 20°, 24° which indicates the crystalline nature of naringin. The X-ray diffractogram of chitosan showed its semi-crystalline nature. The characteristic peaks of chitosan were at 9.9° and 20°. The X-ray diffractogram of TPP showed its crystalline nature. The sodium tripolyphosphate showed intense peak at 20°, 22°, 25°, 38° and 39°. The X-ray diffractogram of chitosan nanoparticle showed its amorphous nature. It showed characteristic peak at 21°. The peak which was seen in chitosan initially was disappeared (9.9°), this confirms the amorphisation of chitosan nanoparticle. The X-ray diffractogram of naringin loaded chitosan nanoparticle showed its amorphous nature. The intense characteristic peaks of the naringin disappeared in the diffractogram of naringin loaded chitosan nanoparticle. This confirms the amorphization of chitosan, amorphization of naringin due to its complete encapsulation into chitosan nanoparticle. It resulted in the loss of structural arrangement of the lattice during its entrapment. Amorphization in the solid state increases the solubility and bioavailability of the drug. This property makes it beneficial for drug targeting.

Anti-inflammatory activity

Heat induced hemolysis: The naringin and naringin loaded chitosan nanoparticle was effective in inhibiting heat induced hemolysis at different concentrations. It showed the maximum inhibition of

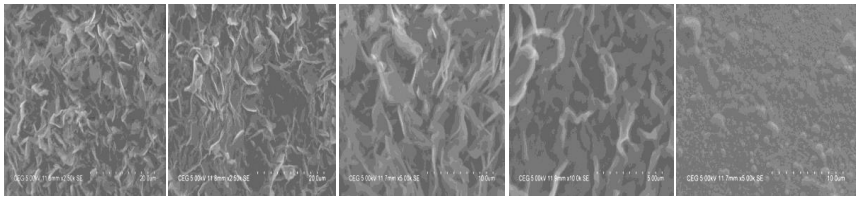

Figure 2: SEM images of Chitosan nanoparticle (CN) at various concentrations and Naringin loaded chitosan nanoparticle (NCN).

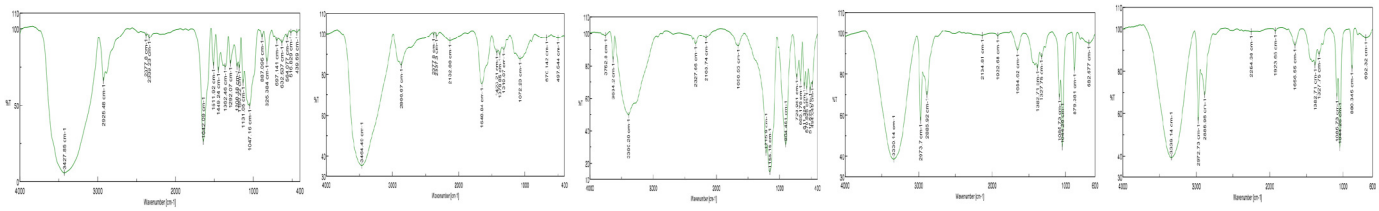

Figure 3: FTIR spectra of Naringin, Chitosan, Sodium Tri Poly phosphate (TPP), Chitosan Nanoparticle (CN), Naringin loaded chitosan nanoparticle (NCN).

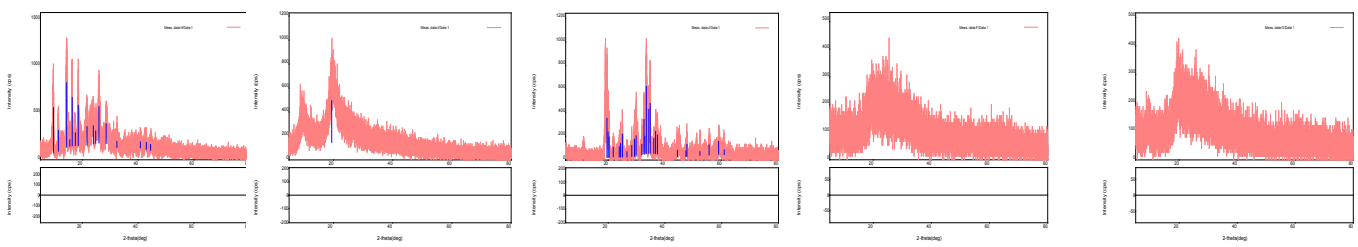

Figure 4: XRD patterns of Naringin, Chitosan, Sodium Tri Poly phosphate (TPP), Chitosan Nanoparticle (CN), Naringin loaded chitosan nanoparticle (NCN).

99.7% at 20 µl, which was at the lowest concentration of naringin. Aspirin was used as the standard drug showed the maximum inhibition of 87%. *O. corniculata* extract was checked for the anti-inflammatory activity, which showed maximum inhibition of 75.75% at 800 µg/ml (Table 1) [6].

Inhibition of albumin denaturation: Effect of naringin and naringin loaded chitosan nanoparticle on albumin Denaturation (Table 2).

A well-documented cause of inflammation is denaturation of proteins. On investigating the mechanism of anti-inflammatory property, the ability of naringin and naringin loaded chitosan nanoparticle was studied. Maximum inhibiton 93% was observed at 16 µl of naringin loaded chitosan nanoparticle. Which confirms the effect of drug is better when incorporated into a nanoparticle (Table 2).

Topic	Concentration	Heated	Unheated	%Inhibition
Control	Negative	0.34	0.034	
Naringin	2 µl	0.009	0.007	99.4%
	4 µl	0.009	0.008	99.7%
	8 µl	0.009	0.008	99.7%
	16 µl	0.009	0.008	99.7%
Naringin Loaded Chitosan Nanoparticle	16 µl	?	0.008	99.7%
Aspirin	250 µg/ml	0.074	0.034	87%

Table 1: Effect of naringin and naringin loaded chitosan nanoparticle on membrane stabilization.

Topic	Concentration	Absorbance	%Inhibition
Control		0.13	
Naringin	2 µl	0.047	63.8%
	4 µl	0.043	66.9%
	8 µl	0.025	84.6%
	16 µl	0.018	86.15%
Naringin Loaded Chitosan Nanoparticle	2 µl	0.048	63%
	4 µl	0.045	65.3%
	8 µl	0.013	90%
	16 µl	0.009	93%

Table 2: Effect of naringin and naringin loaded chitosan nanoparticle on albumin Denaturation.

Anti-coagulant activity

Determination of prothrombin time (PT): The naringin loaded chitosan nanoparticle (100 µM Naringin +0.5% chitosan nanoparticle), increased the clot time when compared to that of the control. The time was noted from 15 minutes upto 3 hours, the naringin loaded chitosan nanoparticle showed a prolonged time to clot. Hence the drug incorporated into the nanoparticle served as an excellent anti-coagulant agent. *O. corniculata* extracts were effective in inhibiting the heat induced albumin denaturation, which showed maximum inhibition 85.92% at 800 µg/ml [6].

***In vitro* antioxidant activity**

1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging: The highest antioxidant activity was given by naringin loaded chitosan nanoparticle at the concentration of 16 µl IC_{50} value 15.17, when compared to that of naringin at 16 µl, for which the IC_{50} value was 52.6. The highest antioxidant value was given by *Mentha spicata* extract at the concentration of 170 µg/ml (Figure 5 and Table 3).

Cytotoxicity assay: The cytotoxicity assay was carried out using VERO cell lines. It is used to determine the cytotoxicity of naringin and naringin loaded chitosan nanoparticle on Vero cell lines. As the concentration of the naringin increases it was toxic to the cells (Figure 6 and Table 4).

***In-vitro* anti-cancerous activity - HeLa cell line**

Figure 7 shows anti-cancerous activity of naringin and naringin loaded chitosan nanoparticle (NCN) on HeLa cell line.

***In-vitro* anti-cancerous activity-lung cancer cell line (A549):** Naringin and naringin loaded chitosan nanoparticle inhibits the proliferation of cancer cells in both HeLa and Lung cancer cell lines. The HeLa cells showed 37% cell viability and A549 cell showed 30% cell viability. The inhibition was better when naringin was incorporated into chitosan nanoparticle, which proves the drug becomes more effective when it was synthesised as a nanoparticle and shows an effective (Figure 8 and Table 5).

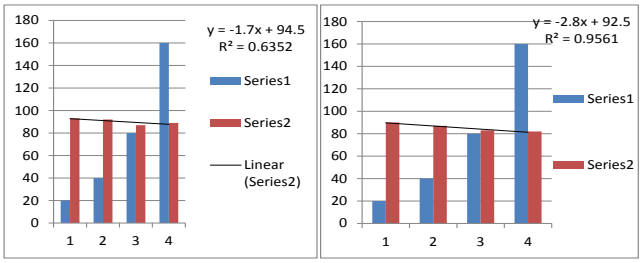

Figure 5: Graph showing IC_{50} value of Naringin IC_{50}=52.6 and Naringin loaded chitosan nanoparticle IC_{50}=15.17.

Topic	Concentration	Absorbance	% Inhibition
Naringin	20 µl	0.022	93%
	40 µl	0.026	92%
	80 µl	0.034	87%
	160 µl	0.041	89%
Naringin Loaded Chitosan Nanoparticle	20 µl	0.036	90%
	40 µl	0.042	87%
	80 µl	0.052	83%
	160 µl	0.058	82%

Table 3: Free radical scavenging activity of naringin and naringin loaded chitosan Nanoparticle.

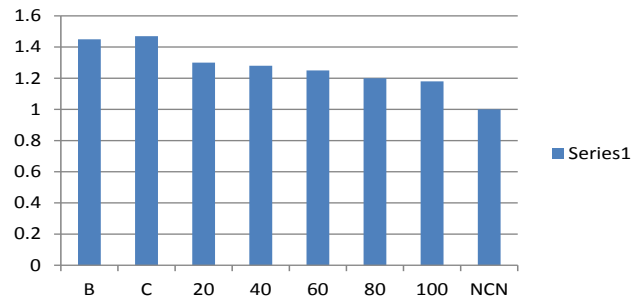

Figure 6: Graph showing cytotoxicity activity of naringin and naringin loaded chitosan nanoparticle (NCN) on Vero cell line.

Sample	%Cell viability
NARINGIN	60%
NARINGIN LOADED CHITOSAN NANOPARTICLE	50%

Table 4: Cytotoxicity assay.

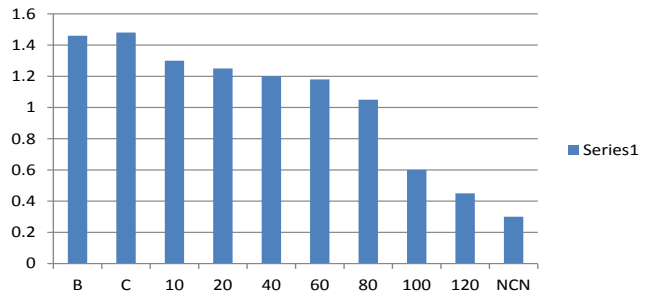

Figure 7: Graph showing anti-cancerous activity of naringin and naringin loaded chitosan nanoparticle (NCN) on HeLa cell line.

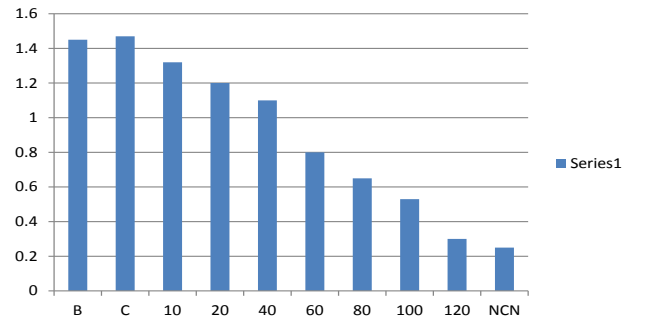

Figure 8: Graph showing anti-cancerous activity of naringin and naringin loaded chitosan nanoparticle (NCN) on Lung cancer cell line (A549).

Test sample	Cell line	% Cell viability
Naringin+NCN (100 µM)	HeLa	37%
Naringin+NCN (100 µM)	Lung cancer (A549)	30%

Table 5: The percentage viability of cancer cells on treatment with naringin and naringin loaded chitosan nanoparticle.

Cell counting

The total count of cells obtained after the trypsinising the mouse mesenchymal stem cell lines were 4.6×10^3 cells/µl. Thus 1 ml of suspension contained 4.6×10^6 cells (Figure 9).

Characterization assays

MTT assay for cytotoxicity analysis: The MTT assay (Figure 10) showed the drug, naringin and naringin loaded chitosan nanoparticle were not toxic to the cells. The growth curve showed an increase in MTT value at a dose of naringin (20 µM, 40 µM, 60 µM, 80 µM and 100 µM) and naringin loaded chitosan nanoparticle (100 µM). The color intensity and the absorbance value increased as the cell viability increases. The absorbance value of control was found to be less when compared to the other values, as it does not contain any drug. The concentration of naringin (20 µM) increased the cell viability when compared to control. On further addition of chitosan nanoparticle to 100 µM of naringin there was a further increase in the cell viability. In a similar way the maximum dose of naringin 100 µM and 0.5% chitosan nanoparticle showed maximum cell viability and this concentration was hence proved to be nontoxic to the cells. When human mesenchymal stem cell was treated with paclitaxel there was a uniform reduction in cell number, even though there was no appreciable difference between treatment concentrations.

Alkaline phosphatase assay: The BCIP-NBT method showed that the alkaline phosphatase activity was increased in 20 µM naringin (Figure 11) when compared to the positive and negative control on day 10.

A high dose of naringin and naringin loaded chitosan nanoparticle in the concentration 100 µM and 0.5% was found to increase the alkaline phosphatase activity when compared to the other concentrations. Hence, the results obtained imply that naringin and naringin loaded chitosan nanoparticle were highly potential in inducing alkaline phosphatase activity. On comparing osteogenic with basal condition, ALP activity was increased in osteogenic condition. A large number of mesenchymal stem cells committed to the osteogenic lineage were necessary for creating a tissue engineered implant to fill bone defects.

Picrosirius red staining: Collagen deposits in positive control was compared and determined by picrosirius red staining. The results

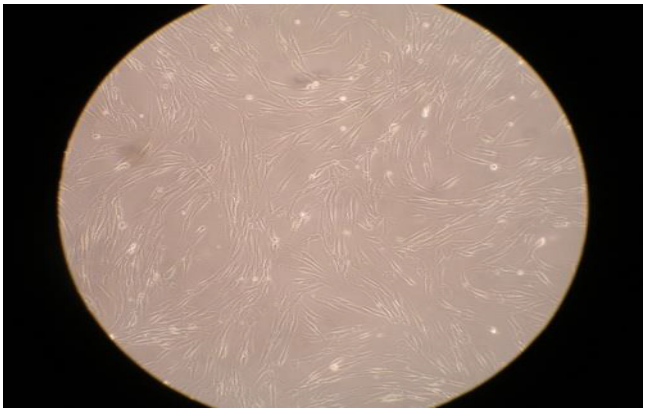

Figure 9: Mouse mesenchymal stem cell line (C3H10T 1/2).

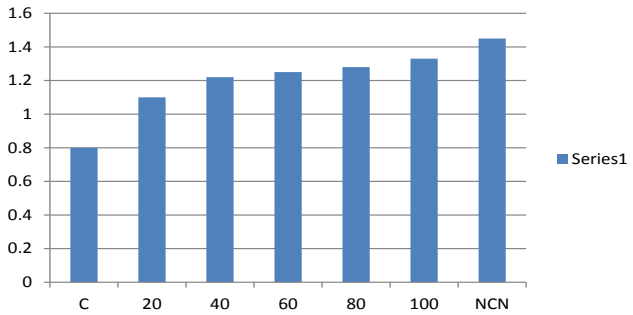

Figure 10: Graph showing MTT assay for cytotoxicity analysis.

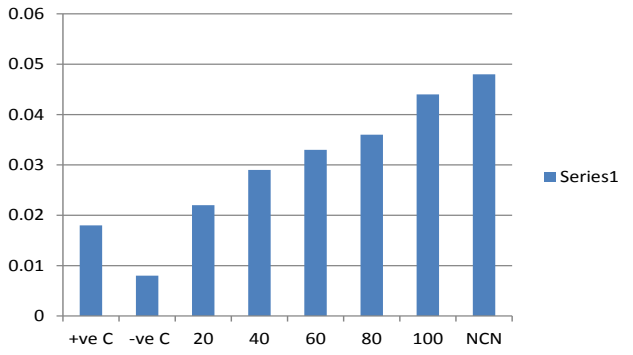

Figure 11: Graph showing alkaline phosphatase assay for various concentrations of naringin and naringin loaded chitosan nanoparticle (NCN).

showed increased collagen deposition in 100 μM naringin and 0.5% chitosan nanoparticle when compared with the positive control (Figure 12).

Further, the collagen deposits were present uniformly throughout the different concentrations of naringin and naringin loaded chitosan nanoparticle and also in positive control. These results indicate the increased osteogenic properties on addition of the drug and chitosan nanoparticle. The Picrosirius red staining of LV tissue was compared as BMC, medium group,Dox group, in which the BMC(Bone Marrow Cells) showed mildly high deposition of collagen when compared with the others.

Alizarin red staining: The calcium deposition was determined by alizarin red s staining. The amount of calcium deposited in different concentrations of drug and chitosan nanoparticle were compared with positive and negative control.

The results showed increased calcium deposition in maximum concentration of naringin and naringin loaded chitosan nanoparticle compared with positive control (Figure 13). The calcium was deposited in form of crystals after mineralization uniformly throughout the cells with various concentrations of naringin and naringin loaded chitosan nanoparticle and the positive control. These results indicate the mineralizing and osteogenic properties of naringin.

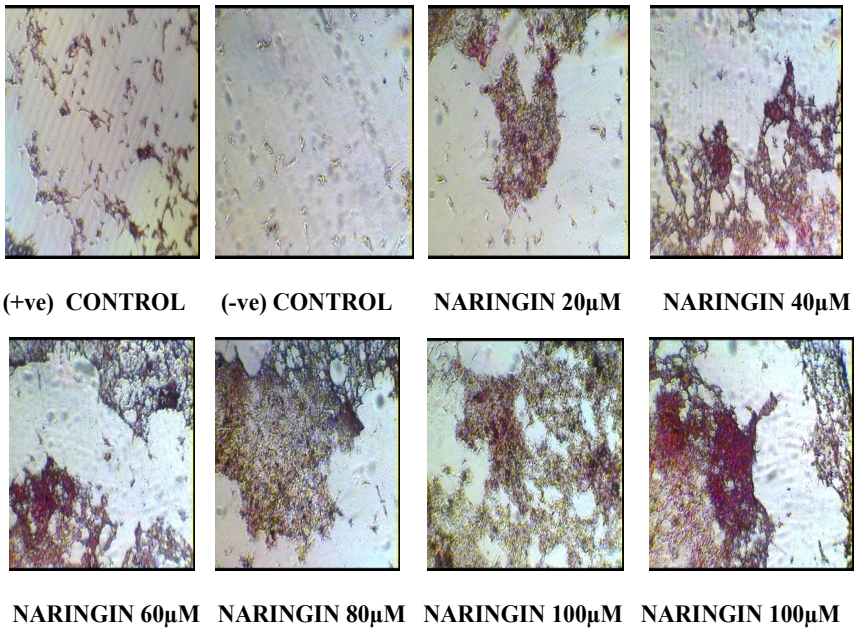

Figure 12: Collagen depositions in mouse mesenchymal stem cell line at different concentration of naringin and naringin loaded chitosan nanoparticle.

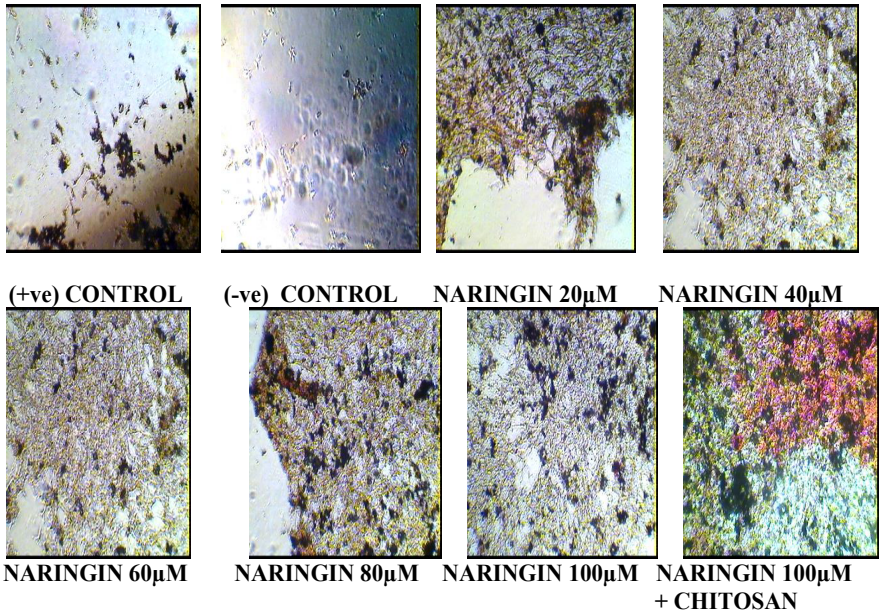

Figure 13: Calcium depositions in mouse mesenchymal stem cell line at different concentration of naringin and naringin loaded chitosan nanoparticle.

When Plasma and Fetal calf serum were provided as a supplement to the mesenchymal stem cells, it showed increased calcium deposition, which can be used in bone repair.

Conclusion

The aim of the present study was to synthesise the Naringin loaded chitosan nanoparticle for bone regeneration. The results indicate that naringin and naringin loaded chitosan nanoparticle can not only promote the secretion of bone formation proteins (BMP) but also enhance the proliferation and osteogenic differentiation of BMSCs. Many herbs are applied in the treatment of osteoporosis and bone non-union, in traditional Chinese medicine. Due to their complicated components, it is very hard to find an effective monomer and arrive at convincing results to prove their therapeutic functions. Exploration about the effect of these raw materials main effective component on the osteogenic differentiation of BMSCs will be helpful as well.

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