Electrospun Polycaprolactone Fibers Loaded with Naringin for Bone Tissue Engineering: Effect of Polycaprolactone and Naringin Concentrations on Cumulative Naringin Release

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Abstract

Electrospun fibers with tunable structural and drug delivery ability have attracted increasing attention in various biomedical applications, especially in tissue engineering. Naringin, flavanone glycoside having excellent osteogenic and pro-angiogenic properties which is highly desired for bone regeneration. In this study, naringin loaded polycaprolactone fibers were fabricated with different concentrations of polycaprolactone and naringin. The scanning electron microscopy results showed that increasing polycaprolactone concentrations increased fiber diameters. Also, varying concentrations of naringin showed an additional increase in fiber diameter compared to control. Further, drug release study showed an increased cumulative naringin release with increasing fiber diameters for 12 days. X-ray diffraction analysis indicated amorphization of naringin in fibrous mats. Also, Fourier transform infrared spectroscopy analysis confirmed naringin was not chemically altered during fabrication process. Furthermore, osteogenic potential of naringin loaded polycaprolactone fibers was evaluated with C3H10T1/2 cell line for 12 days. von Kossa and picrosirius red staining suggested an increased calcium and collagen formation respectively in naringin loaded polycaprolactone fibers. These results suggest that increasing naringin concentrations, increases fiber diameter and consequently cumulative release of naringin. Hence, varying polycaprolactone and naringin concentrations could act as a tool to alter cumulative naringin release within advantageous ranges for bone tissue engineering applications.

Keywords

Electrospinning; Polycaprolactone Fibers; Naringin; Drug Delivery; Bone Tissue Engineering

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INTRODUCTION

Controlled drug delivery systems has aroused increasing attention of biomedical researchers due to its advantages such as reduced drug administration frequency and effective therapeutic potential [1–3]. Numerous studies have been reported that controlled delivery of various bioactive molecules using different forms of micro/nanostructures from many different natural and synthetic polymers [4–6]. Among these, electrospun fibers have enormous applications in biomedical research, which includes controlled drug delivery systems for various tissue engineering applications [7,8]. Moreover, electrospun fibrous mats are preferred in tissue engineering applications due to its advantages such as high surface-area-to-volume ratio, cost-effectiveness, high loading capacity, multi-drug delivery *etc* [9–11].

Polycaprolactone is Food and Drug Administration approved polymer having properties such as biocompatibility, biodegradability *etc* [12,13]. These properties facilitate its use as drug carriers for sustained release in various tissue engineering applications such as bone tissue engineering, sutures and wound dressing, nerve regeneration, cardiovascular tissue engineering *etc* [13]. Due to slower degradation rate, polycaprolactone has been widely studied for long term implants to deliver the encapsulated bioactive molecules in sustained manner [14,15].

Naringin is a flavanone glycoside found in grapefruit and related citrus species [16,17]. Several studies have reported the therapeutic potential of naringin ranging from anti-inflammatory, antioxidant, antiulcer, anticancer properties *etc* [18,19]. In addition, naringin promotes proliferation and osteogenic differentiation of human bone marrow derived mesenchymal stem cells [20]. Previous study reported that, naringin loaded polycaprolactone and polycaprolactone-poly(ethylene glycol)-block-polycaprolactone fibers enhanced mineralization in MC3T3-E1 osteoblasts cell line [21]. Moreover, naringin has reported to promote angiogenesis in diabetic foot ulcer of rats [22]. Hence, naringin being pro-angiogenic and osteogenic molecule could potentially accelerate bone healing which is highly desired in bone regeneration.

Electrospinning is a straight forward technique to fabricate the fibers with diameters ranging from nano to micro which is hard to synthesize by standard mechanical fiber methods [23]. In addition, it allows us to construct continuous fibers from various natural and synthetic polymers [24,25]. Several pharmaceutical agents such as drugs, small molecules, peptides and proteins have been successfully incorporated into electrospun fibers for site-specific controlled drug delivery applications [26,27]. Several parameters such as solution, processing and ambient parameters can influence fiber morphology in electrospinning process [28].

Among parameters governing electrospinning process, polymer and drug concentrations has greater influence on the fiber morphology and also cumulative release of the drug [29]. Previous studies have reported that increasing polymer concentration resulted in increased fiber diameter [30,31]. However, the effect of various polycaprolactone and naringin concentrations on cumulative naringin release profiles has not been reported earlier. Hence, we proposed that increased polycaprolactone concentrations along with varying concentrations of naringin will immensely aid in obtaining desired naringin cumulative release appropriate for therapeutic applications. In this study, polycaprolactone fibers with naringin at various concentrations were electrospun and their osteogenic differentiation potential were evaluated *in vitro* using C3H10T1/2 mesenchymal cell line. The morphology and chemical characteristics of the fabricated fibers was studied using scanning electron microscopy, X-ray diffraction and Fourier transform infrared spectroscopy analysis respectively.

MATERIALS AND METHODS
Materials

Polycaprolactone, Naringin (≥95% (HPLC grade)), Ascorbic acid, Dexamethasone, β-glycerophosphate, 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium (BCIP/NBT) Liquid Substrate System, Hematoxylin, Direct Red 80 were purchased from Sigma-Aldrich (St. Louis, MO). Dulbecco's Phosphate Buffered Saline (DPBS), Dulbecco's Modified Eagle Medium-Low glucose (DMEM-LG), Antibiotic Antimycotic solution, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyltetrazolium bromide (MTT), Trypsin-Ethylenediaminetetraacetic acid (Trypsin-EDTA) solution, Silver nitrate, Picric acid were purchased from Himedia, India. Fetal Bovine Serum (FBS) were purchased from PAN Biotech, Germany. All other chemicals and solvents used were of analytical grade.

Preparation of naringin loaded polycaprolactone fibers

The fibers were obtained by an electrospinning process using ESPIN – NANO (PECO – Chennai, India). Polycaprolactone (10% (w/v) and 12% (w/v)) solutions were prepared by dissolving the polymer in equal volumes of dichloromethane and dimethylformamide [32]. Varying concentrations of

naringin were added to the prepared polycaprolactone solutions as described in Table 1 and transferred to syringe fitted with a needle (0.45x13mm). Fibers were obtained by applying a

high voltage of 15 kV, flow rate of 2 ml/hr, collector drum speed of 1500 rpm and 15 cm distance from the needle tip to collector.

Table 1: Polycaprolactone and naringin concentration parameters for electrospinning

Groups	Concentrations	
	Percentage of Polycaprolactone Solution (w/v)	Naringin (mg/ml)
10%PCL	10	0
10%PCL-Nar3mg/ml	10	3
10%PCL-Nar6mg/ml	10	6
12%PCL	12	0
12%PCL-Nar3mg/ml	12	3
12%PCL-Nar6mg/ml	12	6

Characterization of the fabricated fibers
Scanning electron microscopy (SEM)
To observe the fiber diameter, fabricated fibers were cut into small pieces along with aluminium sheet and sputtered with gold. Then they were analyzed by SEM (TESCAN VEGA3 SBU) with an applied voltage of 10 kV and 5000x magnifications. The average fiber diameter for all groups (Table 1) were calculated manually by measuring 30 fibers, selected randomly from each SEM image using ImageJ software (ImageJ 1.51j8, National Institutes of Health, USA) and the results were expressed as mean±standard deviation.

Naringin release from naringin loaded polycaprolactone fibers
To determine the naringin release, fibers (1cm×1cm) were incubated in 2 ml of DPBS under static condition [32]. Further, 700 µl was aliquoted at regular time intervals and simultaneously replaced with equal volume of DPBS. The absorbance of naringin was read at 284 nm using an UV-Vis spectrophotometer (Systronics, India) and the concentration of drug released was calculated using the standard curve.

Fourier transform infrared spectroscopy (FTIR)
The fabricated fibers and naringin were examined by FTIR spectrophotometer (Perkin Elmer). Samples (3 mg) were mixed with 300 mg of potassium bromide (KBr) and the pellets were made with mechanical pellet maker. Further, the pellets were analyzed in FTIR spectrophotometer operating at 4,000–400cm^{-1} with 1cm^{-1} resolution.

X-ray diffraction (XRD)
The fibers and naringin were subjected to X-ray diffraction using PANalytical X'Pert PRO Powder with a current of 40mA and a load of 45 kV tension. The 2θ scan ranging from 5°–50° with a step size of 0.05.

Cell culture
Mouse mesenchymal cell line, C3H10T1/2 was purchased from National Centre for Cell Sciences (NCCS), Pune, India. The cells were grown in undifferentiated state using complete culture medium (DMEM-LG supplemented with 10% FBS and 1% antibiotic-antimycotic) under standard culture conditions (5% CO_2 humidified incubator at 37°C). At 80% confluence the cells were trypsinized and subcultured using trypsin-EDTA solution [33,34].

In vitro osteogenic differentiation
Cells (5 x 10^4) were seeded in groups in 12 well plates named as control (12%PCL) and Nar-PCL (12%PCL-Nar3mg/ml) containing complete culture medium and incubated overnight under standard culture conditions to facilitate the cell attachment. The 12%PCL-Nar3mg/ml group was preferentially choosen for osteogenic analysis due to its desired cumulative release of naringin. Further, fibers (1cm×1cm) were sterilized using graded series of ethanol and exposed to ultraviolet light for 30 minutes followed by rinsed with DPBS. To the attached cells, fibers were added to the respective groups and cultured in osteogenic differentiation medium (DMEM-LG containing 10% FBS, 1% antibiotic-antimycotic solution, 50 µg/ml ascorbic acid, 10 nM dexamethasone and 10 mM β-glycerophosphate) under standard culture conditions for 12 days with a change of medium every three days. To evaluate the cytotoxicity and osteogenic potential of fibers the following assays were performed.

Cytotoxic evaluation of Nar-PCL
MTT assay was carried out to evaluate the cytotoxicity of Nar-PCL [35]. On day 3, the wells were washed with sterile DPBS and then MTT solution (0.5 mg/ml in DPBS) was added and incubated for four hours to form formazan crystals. After incubation, the MTT solution was removed and 700 µl of DMSO

was added to solubilize the formazon crystals and read at 590 nm using UV-Vis spectrophotometer.

Alkaline phosphatase (ALP) assay

The ALP was quantified using BCIP-NBT liquid substrate solution [36]. On day 7, medium was removed and washed with sterile DPBS. Further, 400 µl of BCIP-NBT solution was added to each well and incubated for 2 hours. After incubation, the violet crystals were solubilized with 600 µl of sodium dodecyl sulfate with 10% HCl solution for 18 hours and read at 595 nm using UV-Vis spectrophotometer.

von Kossa staining

On day 12, von Kossa staining was done to study the calcium deposition [37]. The medium was removed, washed with DPBS and fixed with 10% neutral buffered formalin. Furthermore, wells were stained with 1% of silver nitrate solution, illuminated under ultraviolet lamp for 45 minutes followed by addition of 5% sodium thiosulfate solution to stop the reaction. Then, wells were washed with distilled water and observed under microscope.

Picrosirius red staining

Picrosirius red staining was done to observe the collagen formation [38]. On day 12, the cells were washed with DPBS, fixed with 10% neutral buffered formalin and stained with Weigert's hematoxylin for 8 minutes. Further, staining was done with picrosirius red for one hour and washed with acidified water to remove the excess stain. Collagen formation was observed under the microscope.

Statistical analysis

The results of MTT and ALP assays were statistically evaluated by unpaired student's t-test using GraphPad PRISM 5.01 (San Diego, CA) and the p value (< 0.05) were considered as statistically significant.

RESULTS

Characterization of the fibers

The morphology of fabricated fibers were examined using SEM (Fig. 1A) and interpreted as fiber diameter frequency distribution histograms (Fig. 1B). The results showed that the average diameter of fibers were 311.54±81.50nm and 594.62±226.52nm for 10%PCL and 12%PCL respectively. Similarly, in 10%PCL-Nar3mg/ml and 12%PCL-Nar3mg/ml the average diameters were observed as 332.25±90.46nm and 654.82±337.30nm respectively. Furthermore, the average diameters of

the fibers were 376.57±162.24nm and 715.84±314.91nm for 10%PCL-Nar6mg/ml and 12%PCL-Nar6mg/ml respectively. Overall, the SEM micrographs of fibers showed a dense and smooth morphology.

The naringin cumulative release profiles were examined from the fibers for 12 days. On day 12, the cumulative release of naringin (Fig. 2) was found as 20.01 micromolar (µM) in 10%PCL-Nar3mg/ml, 61.73 µM in 12%PCL-Nar 3mg/ml, 79.92 µM in 10% PCL-Nar6mg/ml and 223.27µM in 12% PCLNar6mg/ml. This study suggests that varying polycaprolactone and naringin concentrations showed an effect on naringin release rates from the fibrous mats.

FTIR spectra revealed characteristic peaks as shown in Fig. 3A. For polycaprolactone fibers, the peaks were observed at 2925.8 cm^{-1}, 2858.2 cm^{-1} which attributed to symmetric CH$_2$- stretching and asymmetric CH$_2$- stretching respectively and 1236.2 cm^{-1} [21,39]. The spectra of naringin showed multiple characteristic peaks at 1647.7 cm^{-1}, 1519.1 cm^{-1}, 1456.8 cm^{-1}, 1363.4 cm^{-1}, 1264.1 cm^{-1}, 1178.6 cm^{-1}, 1137.2 cm^{-1} and 1088.8 cm^{-1} [40]. Also, naringin loaded polycaprolactone fibers showed intense peaks at 1458.6 cm^{-1}, 1364.1 cm^{-1} and 1169.2 cm^{-1} indicating the presence of naringin [40] and the peaks at 2927.1 cm^{-1}, 2861.8 cm^{-1} and 1239.3 cm^{-1} corresponding to polycaprolactone [21,39]. FTIR results indicate that naringin remained chemically unaltered during the fabrication of naringin loaded polycaprolactone fibers.

The XRD study was carried out to determine the crystallinity of fabricated fibrous mats (Fig. 3B). In polycaprolactone fibers, two semi-crystalline diffraction peaks were observed at (2θ) 21.504° and 23.919°. Further, XRD spectra of naringin showed multiple intense peaks at (2θ) 8.095°, 9.053°, 10.144°, 11.918°, 13.416°, 14.928°, 16.884°, 17.789°, 18.780°, 19.879°, 22.194°, 24.362°, 25.237°, 26.466°, 29.047°, 31.139°, 33.093°, 39.471°, 41.216°, 43.024° and 45.003°[41]. Similarly, naringin loaded polycaprolactone fibers also showed two semi-crystaline peaks at (2θ) 21.559° and 23.908° which correspond to polycaprolactone. However, no additional peaks were detected in naringin loaded polycaprolactone fibers which implies that the naringin is amorphized during encapsulation.
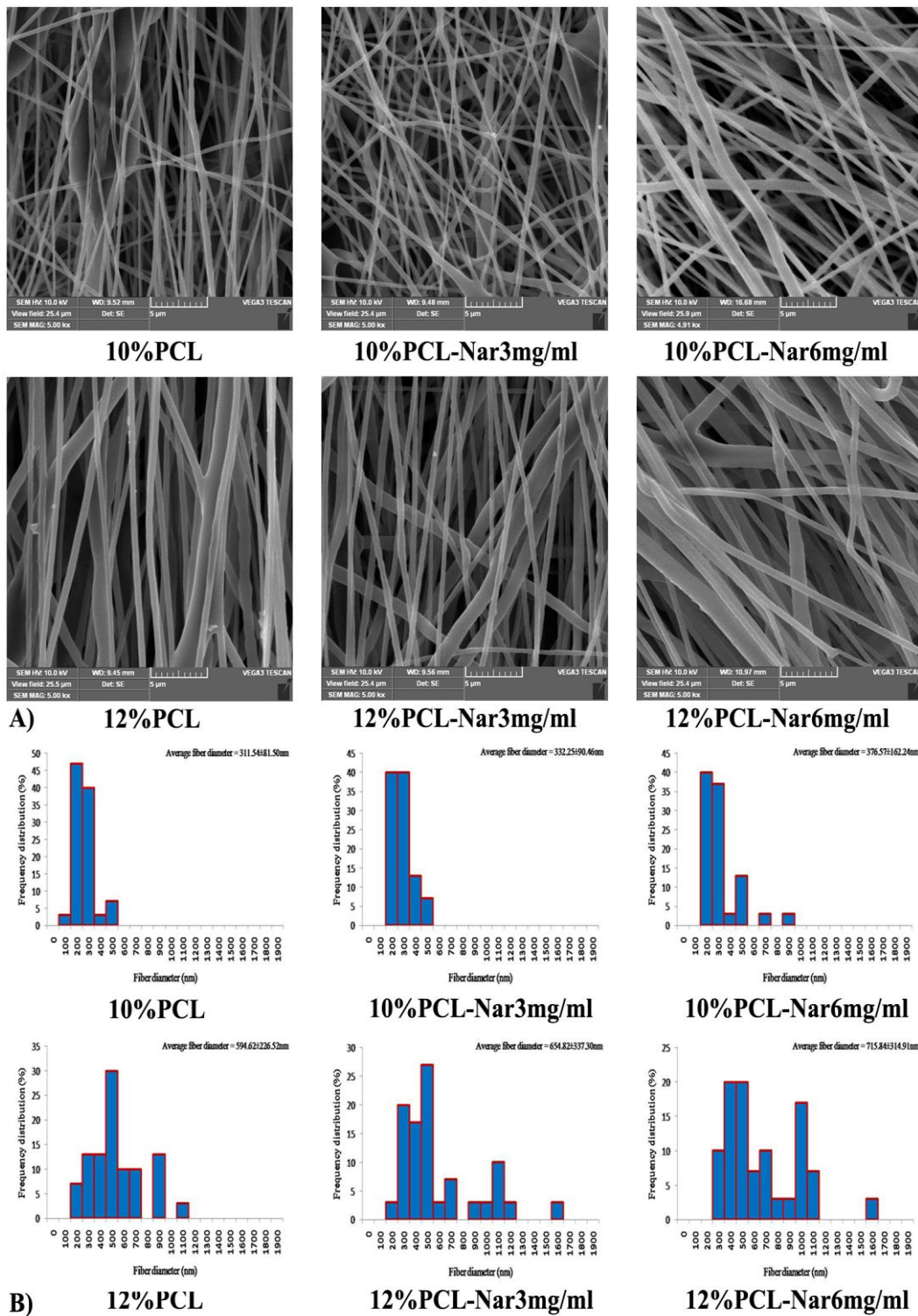
Fig. 1: A) SEM Images showing morphology of the fabricated fibers; B) Fiber diameter frequency distribution histograms. Where, "10%PCL - 10% polycaprolactone fibers"; 10%PCL-Nar3mg/ml - Naringin (3mg/ml) loaded 10% polycaprolactone fibers; 10%PCL-Nar6mg/ml - Naringin (6mg/ml) loaded 10% polycaprolactone fibers; "12%PCL - 12% polycaprolactone fibers"; "12%PCL-Nar3mg/ml - Naringin (3mg/ml)" loaded 12% polycaprolactone fibers; 12%PCL-Nar6mg/ml - Naringin (6mg/ml) loaded 12% polycaprolactone fibers
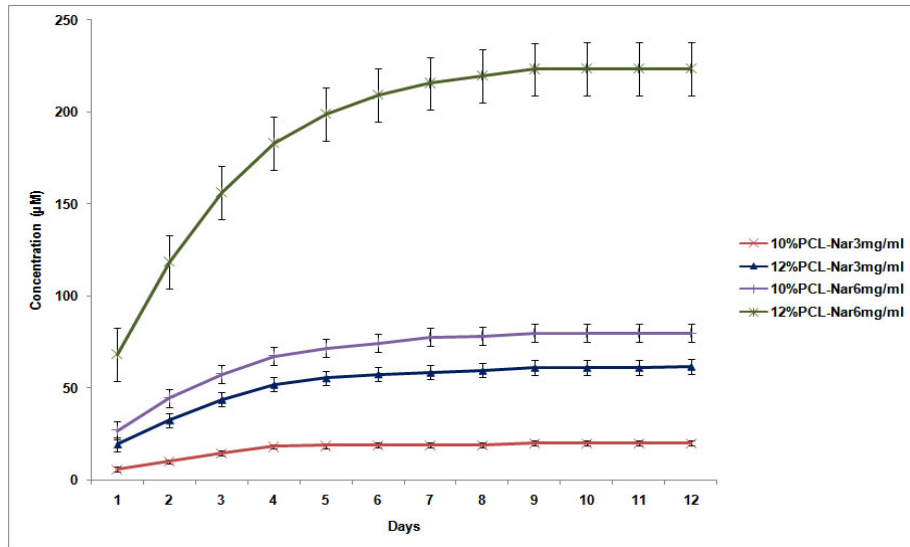
Fig. 2: Cumulative release of naringin from naringin loaded polycaprolactone fibers. Where, 10%PCL-Nar3mg/ml - Naringin (3mg/ml) loaded 10% polycaprolactone fibers; 10%PCL-Nar6mg/ml - Naringin (6mg/ml) loaded 10% polycaprolactone fibers; 12%PCL-Nar3mg/ml - Naringin (3mg/ml) loaded 12% polycaprolactone fibers; 12%PCL-Nar6mg/ml - Naringin (6mg/ml) loaded 12% polycaprolactone fibers
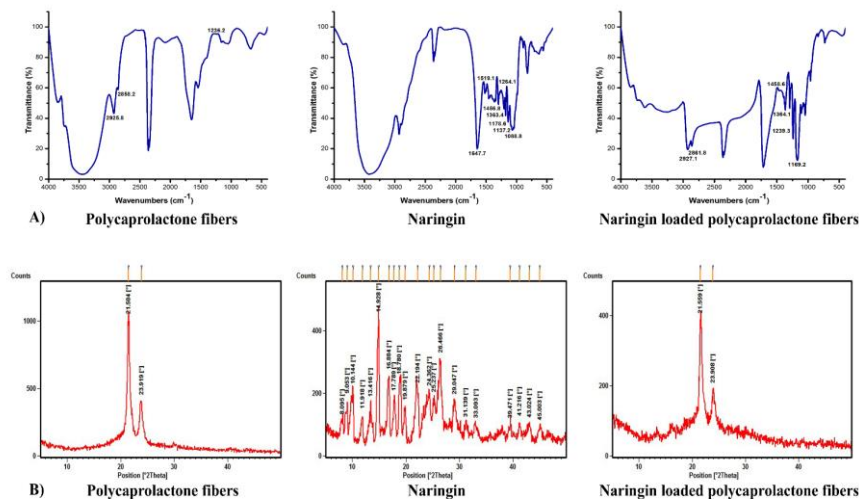

Fig. 3: A) Fourier transform infrared spectra; B) X-ray diffraction spectra
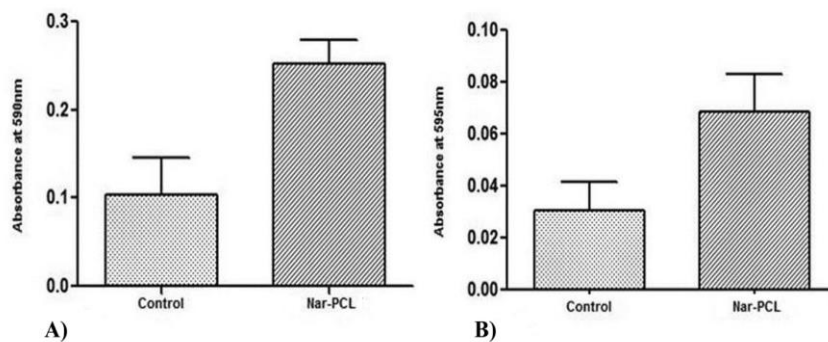

Fig. 4: A) MTT assay; B) Alkaline phosphatase assay.
Where, Control – 12% polycaprolactone fibers; Nar-PCL - Naringin (3mg/ml) loaded 12% polycaprolactone fibers
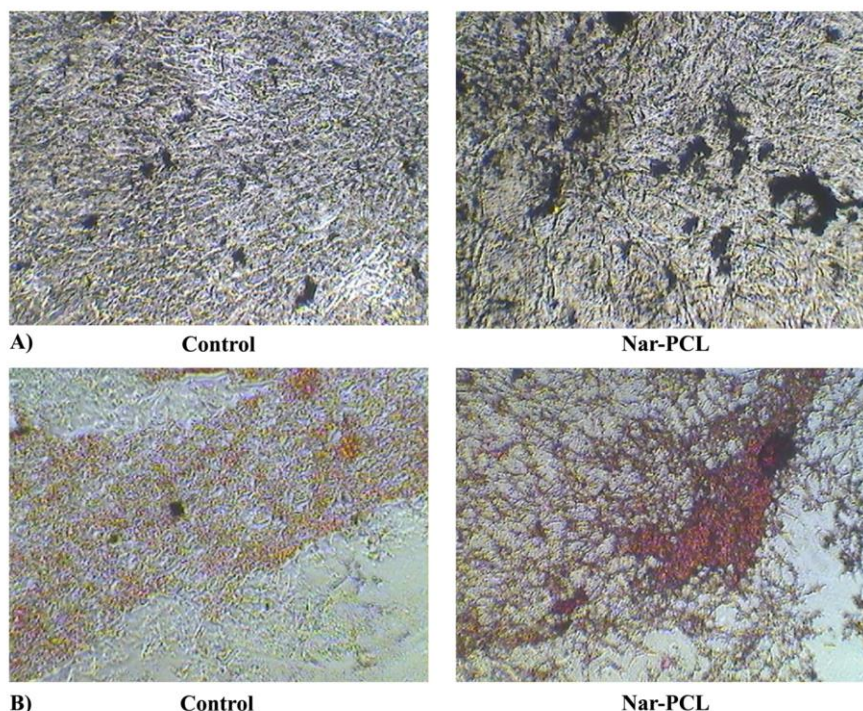
Fig. 5: A) von Kossa staining for calcium; B) Picrosirius red staining for collagen.
Where, Control – 12% polycaprolactone fibers; Nar-PCL - Naringin (3mg/ml) loaded 12% polycaprolactone fibers

Cytotoxic evaluation of Nar-PCL
MTT assay was done to evaluate the cytotoxicity of naringin loaded polycaprolactone fibrous mats. The result implies that the cells were viable and metabolically active on day 3 (Fig. 4A). The absorbance values of Nar-PCL showed significant increase (P< 0.05) suggesting increased metabolic activity and viability in this group.

Osteogenic potential of Nar-PCL *in vitro*
Osteogenic differentiation of Nar-PCL was evaluated using C3H10T1/2 cell line by alkaline phosphatase assay, von Kossa and picrosirius red staining. Alkaline phosphatase is an early marker of osteoblastic cell differentiation [42,43]. The activity of alkaline phosphatase showed a significant increase (P<0.05) in Nar-PCL group compared to control (Fig. 4B). Further, von Kossa and picrosirius red results showed increased calcium deposition (Fig. 5A) and collagen formation (Fig. 5B) *in vitro* respectively. These results suggest that Nar-PCL enhances osteogenesis in C3H10T1/2 cell line.

DISCUSSION
Naringin, a flavanone glycoside has been used to enhance the bone regeneration [20]. To obtain the best suited naringin release range for bone tissue engineering, naringin loaded fibers were fabricated with varying concentrations of polycaprolactone and naringin. In electrospinning process, polymer concentration is one of the important parameter which can potentially affect diameter of the fibers [44]. Also, several studies suggest that increasing polymer concentration results in an increased diameter of fibers [29,44,45]. In this study, the effect of polycaprolactone and naringin concentrations on fiber diameter and consequently its cumulative naringin release were evaluated. The SEM results suggest that the fiber diameter increased considerably with 12%PCL compared to 10%PCL. Also, the average fiber diameters of 10%PCL-Nar6mg/ml and 12%PCL-Nar6mg/ml were larger than 10%PCL-Nar3mg/ml and 12%PCL-Nar3mg/ml respectively, which implies that addition of naringin lead to an increased average fiber diameter compared to control. Similarly, 12%PCL-Nar3mg/ml and 12%PCL-Nar6mg/ml showed increase average fiber diameters compared to both 10%PCL-Nar3mg/ml and 10%PCL-Nar6mg/ml. These results suggest that, in addition to increased fiber diameters with increasing polycaprolactone concentration, varying naringin concentration substantially increased fiber diameter. In addition, the naringin release study indicates that there was a substantial increase in cumulative release of naringin with an

increase average fiber diameters. The results are in accord with previous reports that the diameter of fibers and its cumulative release increased with varying concentrations of drug [9]. The combinatorial effect of varying polycaprolactone and naringin concentration has not been reported earlier. In future, cumulative naringin release can be controlled within desired concentration ranges by titrating crucial factors such as polycaprolactone and naringin concentrations. Further, the FTIR and XRD results indicate that naringin was not modified chemically during the fabrication process, underwent amorphization due to encapsulation. Amorphous form of naringin has increased dissolution hence amorphization is desired in drug delivery applications. Previous studies have reported similar amorphization during encapsulation and its advantages in drug delivery [39,46].

The cytotoxicity assay implies that sustained release of naringin from Nar-PCL increased metabolic activity in C3H10T1/2 cell line and was not cytotoxic. Several studies have reported that, naringin showed an proliferative effect on various cell types, such as human bone mesenchymal stem cells [20], rat osteoblast-like UMR-106 cells [47,48], MC3T3-E1, MG63 [49] and human adipose derived mesenchymal stem cells [50] in a dose-dependent manner. Also, alkaline phosphatase assay results suggest that Nar-PCL enhances the differentiation of C3H10T1/2 cell line to osteogenic lineage. Previous studies have reported an increased alkaline phosphatase activity with dose-dependent naringin concentration in human bone mesenchymal stem cells [20], rat bone marrow stromal cells [51] and human periodontal ligament stem cells [52]. Alkaline phosphatase being an early marker for osteoblast differentiation, the significant increase in alkaline phosphatase activity suggests naringin directs the differentiation of C3H10T1/2 cell line into osteoblast lineage. In addition, increased calcium deposition and collagen formation was observed in Nar-PCL compared to control indicating that naringin enhanced osteogenic differentiation of C3H10T1/2 cell line. Previous study has reported an increased calcium deposition in human bone mesenchymal stem cells [20]. Furthermore, the fibrous morphology has ability to mimic the extra cellular matrix environment to the cells for adhesion thereby increasing potential for bone tissue engineering applications [30,53,54]. In brief, this study emphasizes on the importance of polycaprolactone and naringin concentrations on fiber diameter and its cumulative release, osteogenic potential of naringin loaded polycaprolactone fibrous mats for bone tissue engineering applications.

CONCLUSIONS

In this study, different concentrations of polycaprolactone and varying concentrations of naringin fibers were fabricated. Overall, the results obtained showed that increasing polycaprolactone concentrations has increased fiber diameter considerably. Also, increasing concentrations of naringin increased fiber diameter further, compared to control. Moreover, cumulative concentration of naringin increased with increasing fiber diameters. In addition, naringin loaded polycaprolactone fibers were found to increase osteogenic differentiation in C3H10T1/2 cell line. In summary, this study concludes that the desired cumulative concentration ranges of naringin could be obtained by varying polycaprolactone and naringin concentrations thereby enhancing the therapeutic potential of Nar-PCL in bone tissue engineering applications.

ACKNOWLEDGEMENT

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