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Silymarin-loaded Cellulose Acetate Electrospun Fiber Mats: *In Vitro* Enzymatic Degradability and Cytotoxicity Tests

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ABSTRACT

Silymarin has been widely used as a hepatoprotectant and in supportive treatment of patients with chronic inflammatory liver disorders. Recently, silymarin can be used to protect the skin from oxidative stress induced by ultraviolet (UV) irradiation.In this research, the silymarin-loaded electrospun cellulose acetate (CA) fibers were prepared which silymarin was added in various amounts (i.e., 2.5-20 wt. % based on the weight of CA powder). The biodegradability of prepared 7.5% w/w silymarin-loaded electrospun CA fibers was conducted in phosphate buffer pH 7.4, collagenase type I solution and lysozyme solution. The biodegradability was measured at 2, 4, 6 and 8 weeks. The results found that the CA fiber and silymarin-loaded electrospun CA fibers were not degraded by these test solutions. SEM study of tested fibers after drying with lyophilization technique confirmed the durability of these fibers. Cytotoxicity to human fibroblast cell of the prepared fibers was examined by XTT assay for cell viability value. The human fibroblast cell viability after exposure with 0%, 2.5%, 10%, 20% silymarin-loaded electrospun CA fibers and control was 53.10 ± 2.24%, 64.29 ± 15.36%, 78.01 ± 5.92%, 64.14 ± 5.15%, and 100% respectively. The percentage of cell viability was lower as the silymarin loading was increased. This result implied that fibroblast cell was sensitive with both CA and silymarin

Keywords*: Silymarin, electrospinning, cellulose acetate, cytotoxicity, degradability*

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INTRODUCTION

Polymer fibers are used in a wide variety of applications ranging from textiles to composite reinforcements. Recently, there has been increasing interest in another method of fiber production, i.e. electrostatic spinning or electrospinning, which can produce fibers that are sub-micrometers down to nanometers in diameter. The electrospinning process, first patented by Formhals in 1934 [1], has been studied extensively particularly by Reneker and coworkers [2] and Vancso and coworkers [3] during the early re-visits of the technology. Via careful selections of the solution and the process parameters (e.g. solution concentration, applied electrostatic field strength, etc.), ultrafine fibers with diameters down to a few tens of nanometers could be fabricated.

Cellulose acetate (CA) is one of the most important organic esters derived from cellulose. Liu and Hsieh [4] reported the preparation of ultra-fine CA fiber mats as well as regenerated cellulose membranes by electrospinning. The applications of electrospun CA fiber mat as carriers for topical/transdermal delivery of drugs have been reported [5-7]. It is possible and interesting for using electrospun fiber for topical or transdermal delivery of active compounds. Typically, cellulose can be biodegraded by organisms that utilized cellulase enzymes whereas cellulose acetate molecule comprising additional acetyl group which needs the esterase for the first step in biodegradation.

Silymarin is a group of flavonoids (silibinin, silidianin, and silicristin) that have been isolated from the fruits of *Silybummarianum*. The chemical structure of silymarin was showed in Fig. 1.It has been widely used as a hepatoprotectant and in supportive treatment of patients with chronic inflammatory liver disorders such as cirrhosis, hepatitis, and fatty infiltration due to alcohol and toxic chemicals accumulation. It has also been used in the treatment of patients with liver damage caused by poisonous mushrooms and in the protection of red blood cell membranes against lipid peroxidation and hemolysis (breaking down of red blood cells) caused by certain red blood cell poisons. Recently, silymarin can be used to protect the skin from oxidative stress induced by ultraviolet (UV) irradiation and to treat it [8]. Silymarin products have been supplied in different dosage forms such as capsules, tablets, liquids, powders, and creams [9-10].

The silymarin loaded-CA electrospun fiber mats were prepared in this study. The aim of this research was to develop mats of electrospun CA nanofibers as carriers for delivery of silymarin to the skin. The indirect *in vitro* enzymatic degradability (collagenase type I andlysozyme) and cytotoxicity of as-prepared fiber mats were studied.

MATERAILS AND METHODS

Materials

Cellulose acetate (CA; white powder; Mw = 30,000 Da; acetyl content = 39.7 wt%; degree of acetyl substitution 2.4) was purchased from Sigma–Aldrich (Switzerland). Acetone

and *N, N*-dimethylacetamide (DMAc), were purchased from Labscan (Asia), Thailand. Silymarin was purchased from PanjinHuacheng Pharmaceutical Co., Ltd. Collagenase enzyme Type I (activity 214 Unit/mg) was supplied from Bang Trading Ltd. Dulbecco's Modified Eagle's Medium; DMEM (Sigma-Aldrich Chemie, GmbH, Steinheim, Germany), fetal bovine serum; FBS (Analytical Grade, California, USA), lysozyme (activity > 69,000 Unit/mg, hen egg-white, Sigma-Aldrich, Oakville,Canada) were used as received. Primary fibroblast cells, passage 8 were used in cytotoxicity test. XTT cell proliferation kit (Roche Diagnostics, Indianapolis, USA) was used as received. Sodium hydroxide, NaOH) (Lab-scan Co., Ltd.),potassium phosphate monobasic (Riedel-de Haen Co., Ltd.), sodium chloride (SR Lab, Bangkok, Thailand), sodium phosphate (B/No.AF405300,Ajax Fineche ltd.) were analytical grade.

Methods

Preparation of the neat and the Silymarin-loaded CA fiber mats and films

An initial amount of CA powder was dissolved in 2:1 v/v acetone/DMAc to obtain a 17% w/v CA solution as was stated in the literature [11-12]. Silymarin-loaded CA solutions were prepared by dissolving the same amount of CA powder and silymarin in the amount of 2.5-20% w/w based on the weight of CA powder, respectively, in the acetone/DMAc mixture. These mixtures were stirred for 4 h to obtain the clear solutions.

Electrospinning of the as-prepared solutions was carried out by connecting the emitting electrode of positive polarity from a Gamma High-Voltage Research ES30PN/M692 high voltage DC power supply to the solutions filled in a standard 50-ml syringe, the open end of which was attached to a blunt gauge-20 stainless steel needle (OD = 1.2 mm), used as the nozzle, and the grounding electrode to a home-made rotating metal drum (OD = 12 cm) was covered with aluminum foil to be used as the fiber collection device. Electric potential of 12.5 kV was applied across a fixed distance of 12.5 cm between the tip of the nozzle and the outer surface of the drum. The rotational speed of the rotating drum was 60±5 rpm. The feed rate of the solutions was controlled at 0.1 ml/h by means of a Kd Scientific syringe pump. Electrospinning was carried out at room temperature.

Characterization of the neat and the Silymarin-loaded CA fiber mats

Prior to electrospinning, the as-prepared solutions were measured for their conductivity and shear viscosity using a conductivity meter (Oakton CON 6/TDS 6 Conductivity/TDS meters) and a viscometer (VISCO STAR Plus), respectively. The measurements were carried out at 25°C and average values for each solution were calculated from three measurements.

Morphological appearance of the neat and the silymarin-loaded electrospun CA fiber mats was observed by a JEOL JSM-6380LV scanning electron microscope (SEM). The fiber mats samples were sputtered with a thin layer of gold prior to SEM observation. Diameters of the

individual fibers in the electrospun fiber mats were measured directly from the SEM images using a SemAphore 4.0 software.

In vitro biodegradability test of neat CA and silymarin-loaded electrospun CA fiber mats was investigated in different media. Theprepared fiber was weighed (W1) before placed in 24-well plates containing2.4 mL/well phosphate buffer pH 7.4 (PBS),collagenase type I solution (7.6 μg (28 Unit)/mL in PBS) or lysozyme solution (1.5 μg/mL in PBS). These solutions were removed and filled with the freshly prepared media every week. The tested fibers were removed at different time intervals (2, 4, 6 and8 weeks), washed with distilled water before drying at 50°C and kept in desiccator for 72 h. The %degradability was calculated as following:

$$Degradation\ (\%) \quad = \quad \frac{W_1 - W_2}{W_1} \times 100 \quad \text{.....(1)}$$

where W_1 is the weight of each sample before submersion in different media, W_2 is the weight of the sample after submersion in the different media in its dry state.

Morphological change of the neat CA and silymarin-loaded electrospun CA fiber mats before and after biodegradability was characterized under a scanning electron microscope (Maxim2005, Cam scan, UK). The fiber samples were sputtered with a thin layer of gold prior to SEM observation.

In vitro cytotoxic evaluation was investigated on the primary human fibroblast cells, passage 8 using XTT cell proliferation kit for determination of % cell viability. The neat CA and silymarin-loaded electrospun CA fiber mats were immersed inculture medium (DMEM + 10% FBS) in96 well plastic plate for 12 h before changing theculture medium. Then1 x 10^4 cells/well human fibroblast cells were added onto the test fibers and incubated at 37°C and under 5%CO_2/95% air condition for 24 h. The human fibroblast cells incubated on96 well plastic plates were used as the control group.The cell morphology was observed under the inverted microscope (Nikon, Eclipse TE 2000-S, Japan).After incubation for 24 h, the culture medium was removed and 200 μl DMEM without FBS was added and subsequently the50 μlXTT solution was added into individual well before incubation at37°Cfor4 h. Finally, each well was determined the absorbance at450 nm using microplate spectrophotometer (Spectra Count, Perkin Elmer, Massachusetts, USA) (n=3).

RESULTS AND DISCUSSION

Prior to electrospinning, both the neat and the silymarin-loaded CA solutions were measured for their conductivity and shear viscosity, and results are summarized in Table 1.

The presence of silymarin in the base CA solution was responsible for the increasing of both the viscosity and the solution conductivity, which was according to the silymarin content.

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Such an increased conductivity of the CA solution should be the resulting of dissociation of silymarin into ionic species. Electrospinning of these solutions was carried out at a fixed electric field of 12.5 kV/12.5 cm. Selected SEM images of the electrospun fibers are shown in Fig. 2.

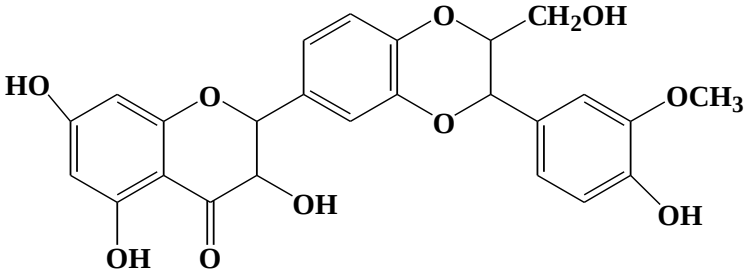
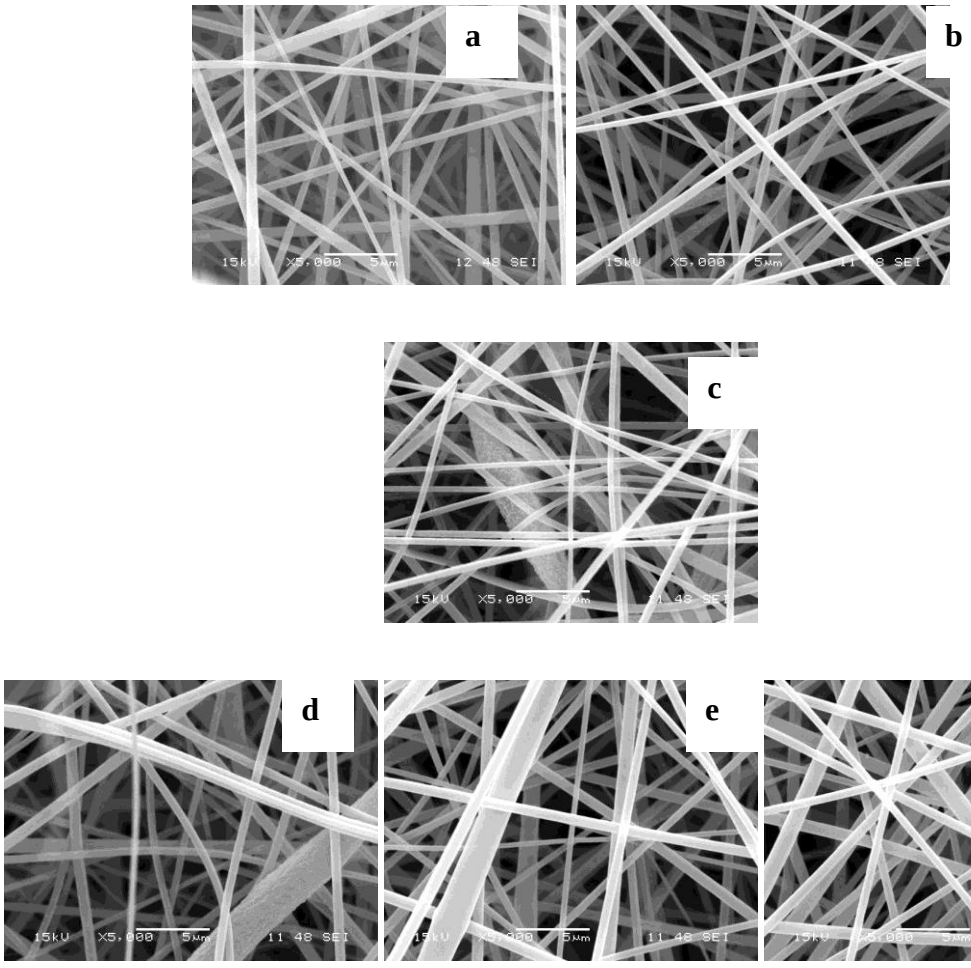


Figure 1 Chemical structure of silymarin.



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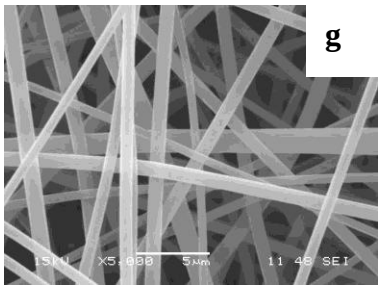

Figure 2 Scanning electron micrographs of the neat and the silymarin-loaded electrospun CA fiber mats at various silymarin contents for (a) 0%; (b) 2.5%; (c) 5%; (d) 7.5%; (e) 10%; (f) 15%; (g) 20%. Note: applied electric field = 12.5 kV/12.5 cm.

Table 1 Electrical conductivity and shear viscosity of the neat and the silymarin-containing CA solutions (n=10) as well as diameters and fiber number of the individual fibers within the resulting electrospun fiber mats (n=100).

Silymarin (% w/w)	Electrical conductivity (µS)	Viscosity (cP)	Fiber diameters (nm)	Fiber number (/100 µm^2)
0	9.07	413.7	608 ± 133	26
2.5	10.96	472.7	573 ± 177	24
5.0	12.82	488.9	551 ± 194	24
7.5	13.75	500.0	623 ± 199	20
10	15.54	540.0	597 ± 173	21
15	18.68	567.7	697± 222	22
20	22.00	596.2	889± 251	21

The round fibers of electrospinning were obtained. Moreover, silymarin aggregates were not found on the surface of these fibers. This might imply that the as-loaded silymarin was perfectly incorporated well within the fibers. The diameters of the neat CA fibers were ~608±133 nm, while those of the silymarin-loaded CA fibers ranged between ~573±177 and 889±251 nm which did not depend on the initial amount of the as-loaded silymarin (see Table 1). However, the diameter of 20% silymarin loaded CA fiber was larger than other silymarin-loaded CA fibers.

The diameter of the as-prepared CA fiber depended on the properties of solution, especially viscosity and conductivity. Normally, the solution viscosity increased with increasing of solution concentration as power-law relationship, and an increased solution viscosity resulted in the formation of fibers of larger diameters [13-14]. Further addition of the silymarin into CA solution enhanced the viscosity of the CA solution and increased the fiber diameter. However, the conductivity of the spinning solution was also increased with the amount of silymarin content. Generally, the increasing in conductivity of the spinning solution caused the decreasing of fiber diameter. Increasing in the number of charge species increased the conductivity, resulting on increase charge density on surface and/or within the polymer jet. Increasing of charge density promoted the greater elongation and thinning the jet as it travels through the electric field onto the collector, which gives smaller fiber diameter.

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Due to the effect of conductivity and shear viscosity of solution on electrospun fiber, the diameter of the silymarin-loaded CA fiber mats was no significant difference with increasing silymarin content. However, 20 %w/w silymarin CA fiber showed the larger fiber, which the shear viscosity of the solution might overcome the conductivity effect, thus the larger diameter fibers were obtained.

The morphology of drug-loaded CA fiber mats depended on type of the drug. It was reported that the vitamin-loaded CA fibers [5], and curcumin-loaded CA fibers [7] were smooth, and no evidence of any kind of aggregation being observed on the surface of the fibers. However, Phiriyawirut and Phaechamud [15] reported that electrospun CA fibers containing 2.5% gallic acid had smooth surfaces, but gallic acid flakes were observed on the fiber surfaces with increasing gallic acid content. This phenomenon depended on the solubility of drug in electrospinning solvent. The smooth surface of fiber was observed when the high solubility of drug in electrospinning solvent. In the other way, the low solubility of drug in electrospinning solvent promoted the flake of drug on the surface of fiber due to aggregation of the undissolved drug particles.

Phosphate buffer pH 7.4, collagenase type I solution and lysozyme solution were used as media for degradation test for as-prepared neat CA and 7.5 %w/w silymarin-loaded electrospun CA fiber mats. The concentration of lysozyme in phosphate buffer was referred to the concentration of lysozyme in human blood [16-18].

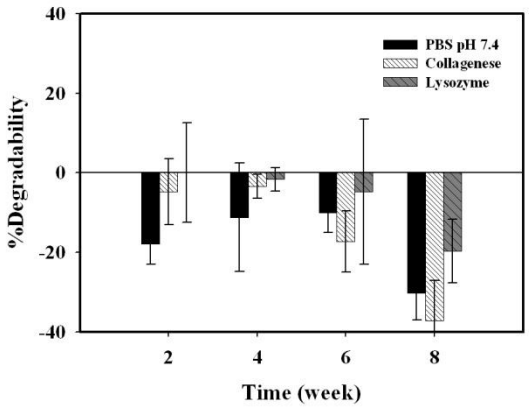

Figure 3Biodegradability of CA fiber mats at different time intervals.

There was the rather low biodegradability of neat CA fiber and silymarin-loaded electrospun CA fiber mats in three media as presented in Figs. 3 and 4, respectively. CA fiber could absorb the aqueous from the used media therefore the minus value was evident for each time interval for three media (Fig. 3). This might have contributed to a hydrogen bonding formation between oxygenorhydroxyl group on CA structure and oxygen or hydrogen of water molecule. Additionally, the high surface/volume or high porosity of fiber promoted the physical absorption and capillary action of water [19]. Loading ofsilymarin promoted small degradation in three media at 2 weeks however, the decreased degradation was found at longer time as

shown in Fig. 4. The release of silymarin at initial stage could decrease the fiber weight and thereafter the water absorption of CA fiber enhanced the fiber weight at the late stage. The SEM study of tested fibers after drying with lyophilization technique confirmed the durability of these fibers from enzymatic degradation (Table 2 and 3). Therefore, silymarin-loaded electrospun CA fiber mats was rather stable for enzymatic degradation. CA has been stated to be degraded with lipase like enzyme produced from some bacteria [20]. It was known that the existence of acetyl-substituted groups in CA inhibited the degradation by cellulase [21-22]. Several research works have been tested for cellulose degradability [23, 24]. The combination of biodegradation and photodegradation allowed a synergy to promote the overall degradation rate [24]. Although it has been reported that the enzyme capable of deacetylation of CA promoted degradation by cellulase [20, 25], only a few lipase or esterase can be effective for degradation of CA [20]. The rather low degradable property of CA fiber denoted its possibility for prolongation the release of the active compounds by mixing with suitable biodegradable polymer as polymer composite electrospun.

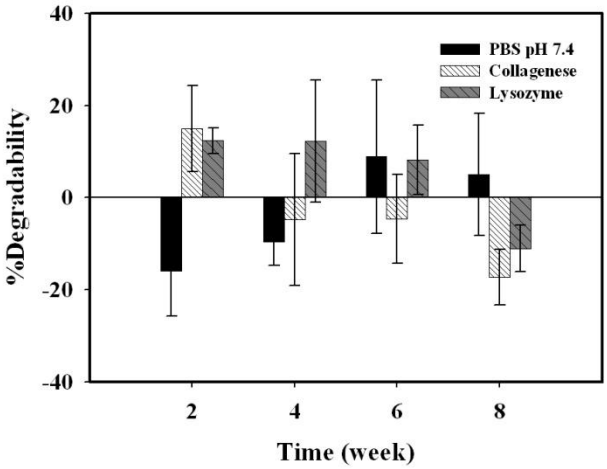

Figure 4Biodegradability of 7.5% Silymarin loaded-CA fiber mats at different time intervals.

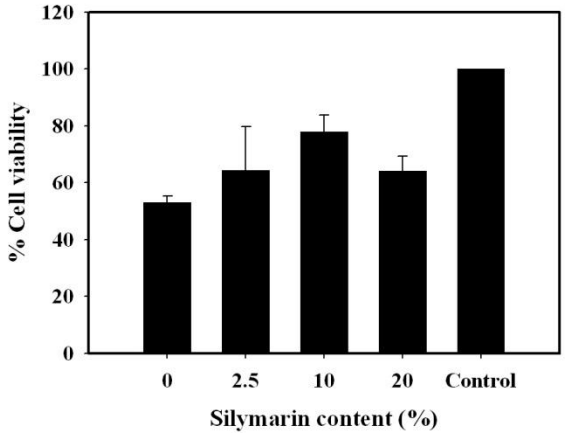

Figure 5 %Cell viability ofhuman fibroblast cells after incubation with CA fiber mats containing different amount of Silymarin for 24 h using fibroblast cells onplastic well plate as control group.

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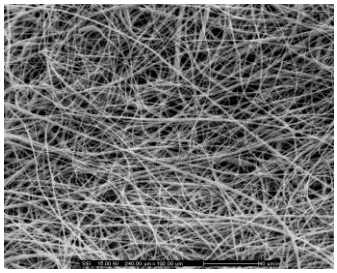
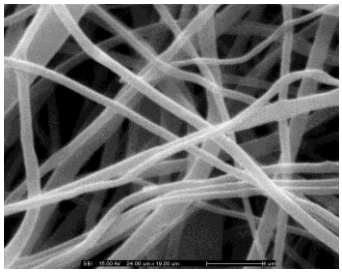
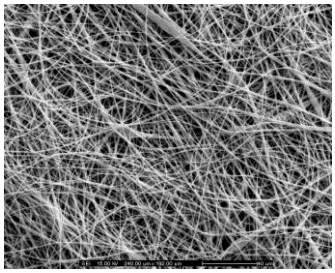
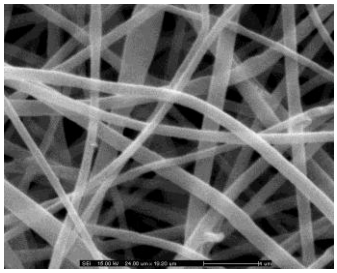
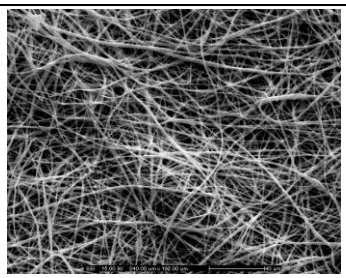
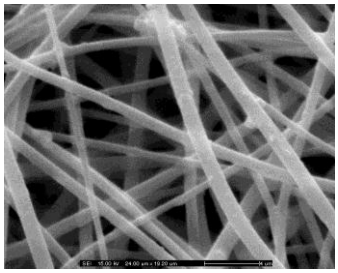
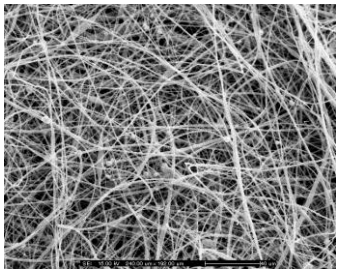
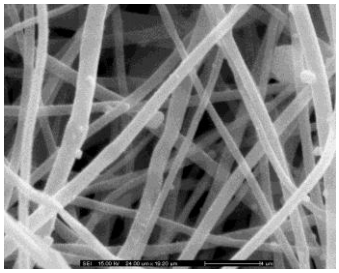
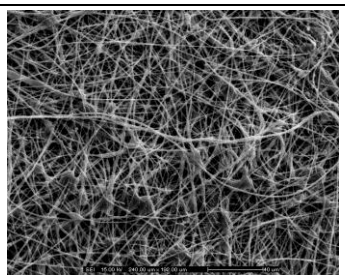
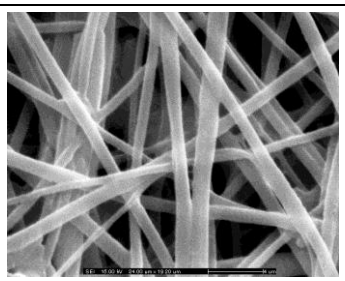
Table 2Morphology of neat CA fiber mats in different type of media after drying with lyophilization technique.

Time (Week)	Media	Magnification	
		500X	5000X
2	Phosphate buffer		
	Collagenase type I		
	Lysozyme		
8	Phosphate buffer		
	Collagenase type I		

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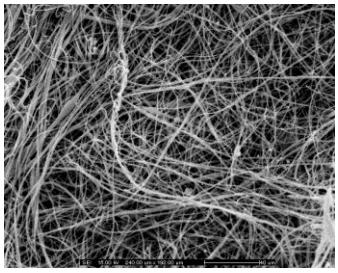
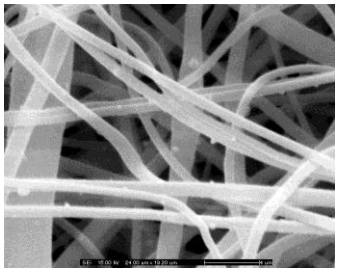
	Lysozyme		

To assess cell compatibility of neat CA and silymarin-loaded electrospun CA fiber mats, primary fibroblast cell was cultured on the prepared fibers. Cytotoxicity of the prepared fibers was examined by XTT assay for cell viability. The morphological structure of test cell did not change after test with CA and silymarin-loaded electrospun CA fiber mats as shown in Table 4. The human fibroblast cell viability of 0%, 2.5%, 10%, 20% silymarin-loaded electrospun CA fibers and control was 53.10 ± 2.24%, 64.29 ± 15.36%, 78.01 ± 5.92%, 64.14 ± 5.15%, and 100% respectively (Fig. 5). The % human fibroblast cell viabilityof silymarin-loaded electrospun CA fiber mats was significantly lower than that of control($p<0.05$). The % cell viability ofneat CA electrospun was lower than that of10 %w/w silymarin-loaded electrospun CA fiber mats, significantly ($p<0.05$). However, this value of 2.5 %w/w silymarin-loaded electrospun CA fiber mats was not significantly different from that of CA electrospun loaded with 20%w/w. This result implied that fibroblast cell was sensitive with both CA and silymarin. Typically, most natural and biodegradable base nanofibers have not shown the cell toxicity [26] and some of them promoting cell growth [27, 28]. Electrospunnanofibers of type 1 collagen, chitosan and polyethylene oxide composite showed no cytotoxicity toward growth of 3T3 fibroblasts [29]. The previous research reported that CA surface promoted the cardiac cell growth and its biodegradability could be controlled by hydrolysis, de-actylization of CA and cytocompatible enzyme action providing glucose as a final product [30]. The prepared electrospun in this study exhibited lower cell viability for human fibroblast cell therefore the test for cell viability to other cells should be confirmed.

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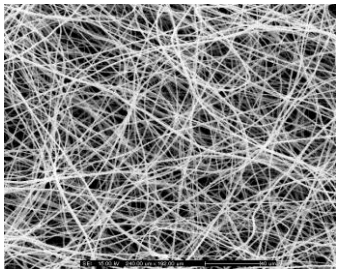
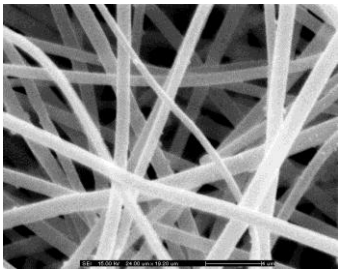
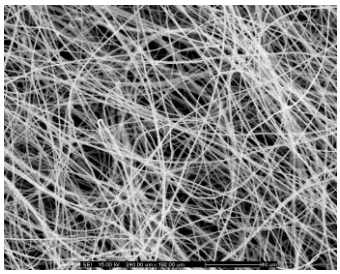
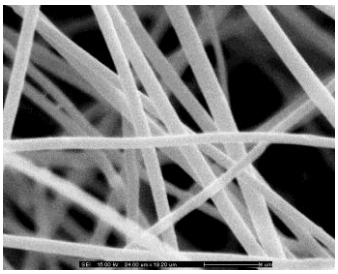
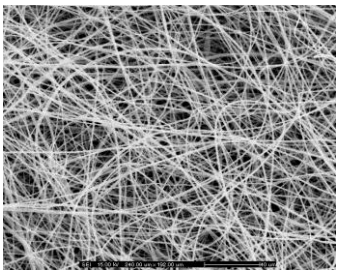
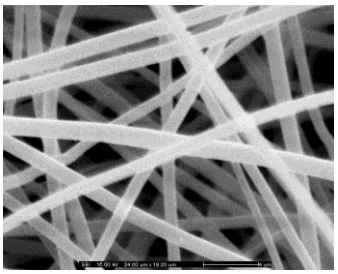
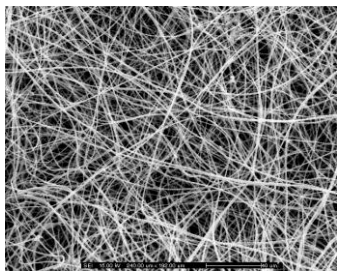
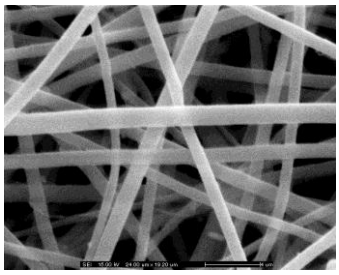
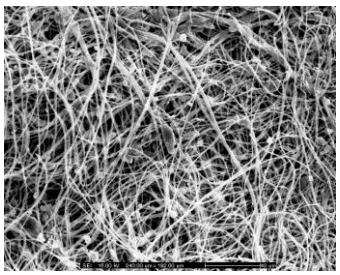
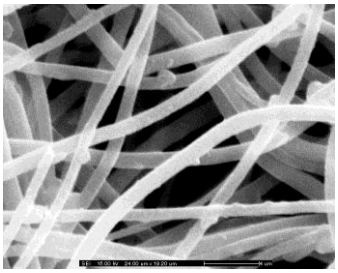
Table 3.Morphology of 7.5% w/w silymarin-loaded electrospun CA fiber mats in different type of media after drying with lyophilization technique.

Time (Week)	Media	Magnification	
		500X	5000X
2	Phosphate buffer		
	Collagenase type I		
	Lysozyme		
8	Phosphate buffer		
	Collagenase type I		

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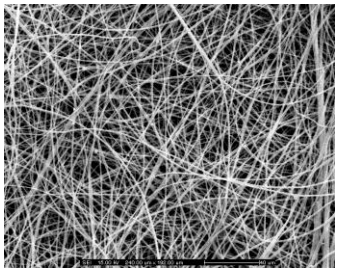
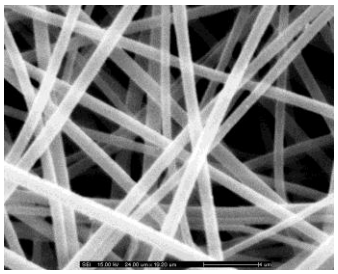
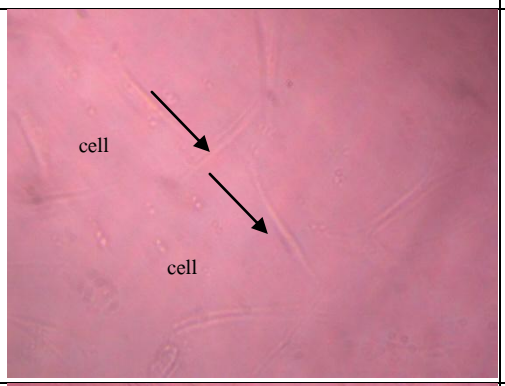
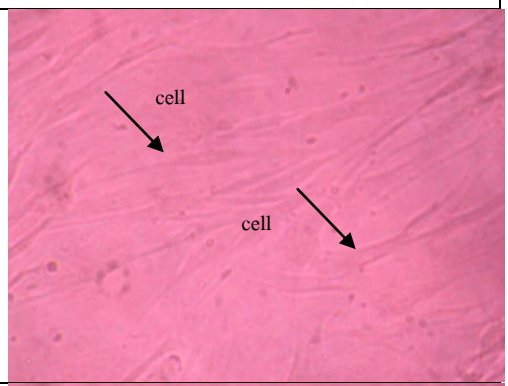
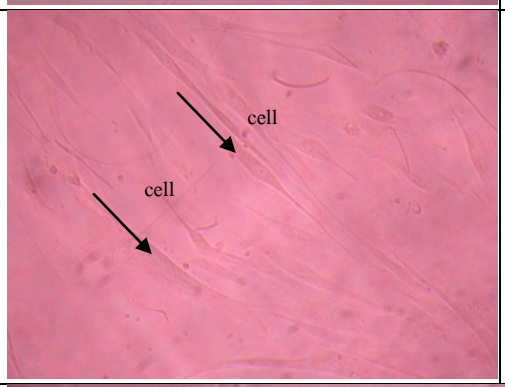
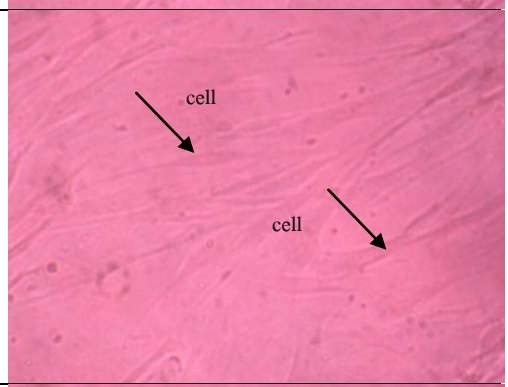
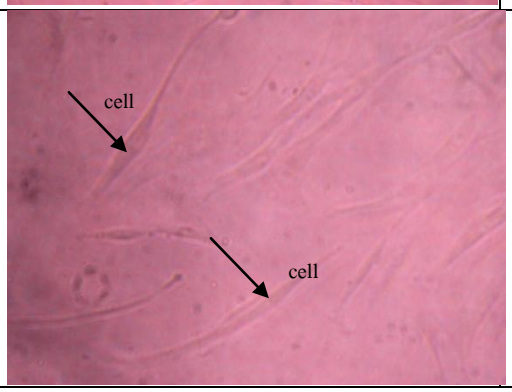
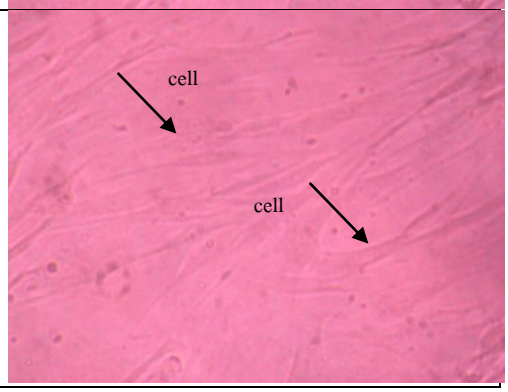
	Lysozyme		

Table 4.Morphology ofhuman primary fibroblast cells after incubation with neat CA fiber mats and silymarin-loaded electrospun CA fiber mats.

Silymarin (% w/w)	20x	Control, 20x
0		
2.5		
10		

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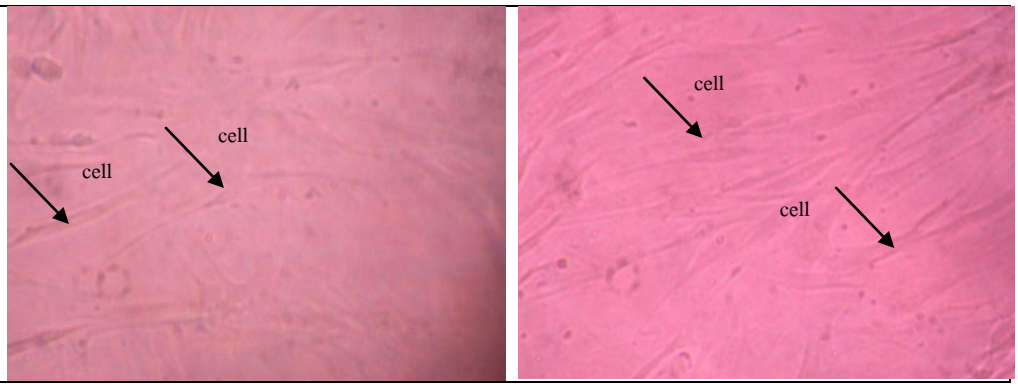
20	

CONCLUSION

Silymarin-loaded electrospun cellulose acetate (CA) fiber mats were prepared by electrospinning technique under a fixed electric field of 12.5 kV/12.5 cm. The 17% w/v CA solution in 2:1 v/v acetone/*N, N*-dimethylacetamide was used as the base spinning solution, into which silymarin at 2.5-20 % w/w (based on the weight of CA) was added to prepare the silymarin spinning solutions. Low biodegradability of neat CA fiber and silymarin-loaded electrospun CA fiber mats was evident in phosphate buffer without or containing collagenase type I or lysozyme. The neat CA and silymarin-loaded electrospun CA fiber mats were rather toxic to the human fibroblast cells. The decreased human fibroblast cell viability was exhibited when the high amount of silymarin was loaded into the CA electrospun fiber mats therefore the further toxicity test to other cells of silymarin-loaded electrospun CA fiber should be performed.

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