CHAPTER IV
RESULTS AND DISCUSSION

4.1 Evaluating MIC and MBC value by Dilution Test and Agar Test

When both *S. aureus* and *E. coli* were tested in each dilution of Ciprofloxacin (CPF), the MIC and MBC were also reported as shown in Table 4.1. The MIC and MBC on *E.coli* is the same value, 0.5 μg/mL, but the MIC and MBC on *S.aureus* is 9.8 μg/mL and 4.9 μg/mL, respectively. Therefore, the quantity of uploaded drug should be least 9.8 μg/mL because it is a minimum concentration which can inhibit the growth of both bacteria, *E.coli* and *S.aureus*. However, the content of CPF at MBC value doesn't mean it can fully inhibit the growth of bacteria when it blended with polymer substrate.

Table 4.1 Minimum inhibitory concentration (MIC) and Minimum bactericidal concentration (MBC) values (μg/mL) of strains used for time-killed study

Microorganism	**Ciprofloxacin content (μg/mL)[a]**	
	MBC	MIC
E.coli	0.5	0.5
S.aureus	9.8	4.9

[a] MIC and MBC values were determined by adding antibacterial agents into nutrient agar.

4.2 Electrospinning Process

The polymeric solution during electrospinning process is continuously elongated, the solvent of polymeric jet is not too evaporated fast, the jet solidifies, and then fibers are formed. Then, the electrospun fibers should be dried in a vacuum dryer for 48 hours or at room temperature in order to remove the solvent out. However, the fibers can be still adhesive in all conditions. As seen in Figure 4.1, the electrospuns of neat PVAc and PVAc with CPF/Oil were detected by electron

microscopy on the surface of fibers. The obtained fibers showed that the incorporation of drug into the fibers reduced the average of diameter distribution of electrospun fibers from 50 μm, pristine PVAc, to 17 μm and 35 μm for PF1.25 and PF2.5, respectively, due to the enhanced conductivity and polarizability of the solution compared to the solution without drug. In term of blend CPF with 5%v/v of coconut oil, they also illustrated the reduction of diameter distribution at the same time which decreased from 50 μm of pristine PVAc to 23 μm and 37 μm for PFO1.25-5 and PFO2.5-5, respectively. On the other hands, the electrospun fibers of PVAc combined with 10%v/v of coconut oil increased the diameter of fibers which reached to 50 μm and 54 μm for PFO1.25-10 and PFO2.5-10, respectively. The reasons why the diameter increased are decreasing conductivity and polarizability while multiplying the oil volume which polymer did not contain enough free electric charges that can carry electrons to facilitate the flow of current within it. When the polymer solution was ejected out onto the substrate, it was harder to stretch to form small fibers. Moreover, the surface of electrospun fibers in SEM image looked rough and coarse when the oil volume is enhanced.

4.3 Bacterial Culture Evaluations

4.3.1 Zone Inhibition

After the results from optimization the uploaded drug, the 9.8 μg/mL of CPF which is a minimum bactericidal concentration, the content of drug is varied to be double as much as the MBC value. The inhibition zone showed the bactericidal activity in *E.coli* and *S.aureus*. The results as shown in Figure 4.2, the killing zone was clearly appeared when the content of ciprofloxacin is 0.31, 0.625, 1.25, and 2.50 mg/mL for *E.coli*. In case of *S.aureus*, the inhibition zone is clear at 1.25 and 2.5mg/mL of CPF. According to the clear zone, *S.aureus* is quite more obvious than *E.coli* so as to resist the CPF because CPF inhibited firstly DNA gyrase and Topoisomerase IV for *E.coli* and *S.aureus*, respectively (Hooper *et al.*, 1987). In contrast, when the ciprofloxacin blended with high oil contents, the clear zone is increased as shown in Figure 4.3. Therefore, coconut oil has enough efficiency to enhance the zone when mixed with CPF.

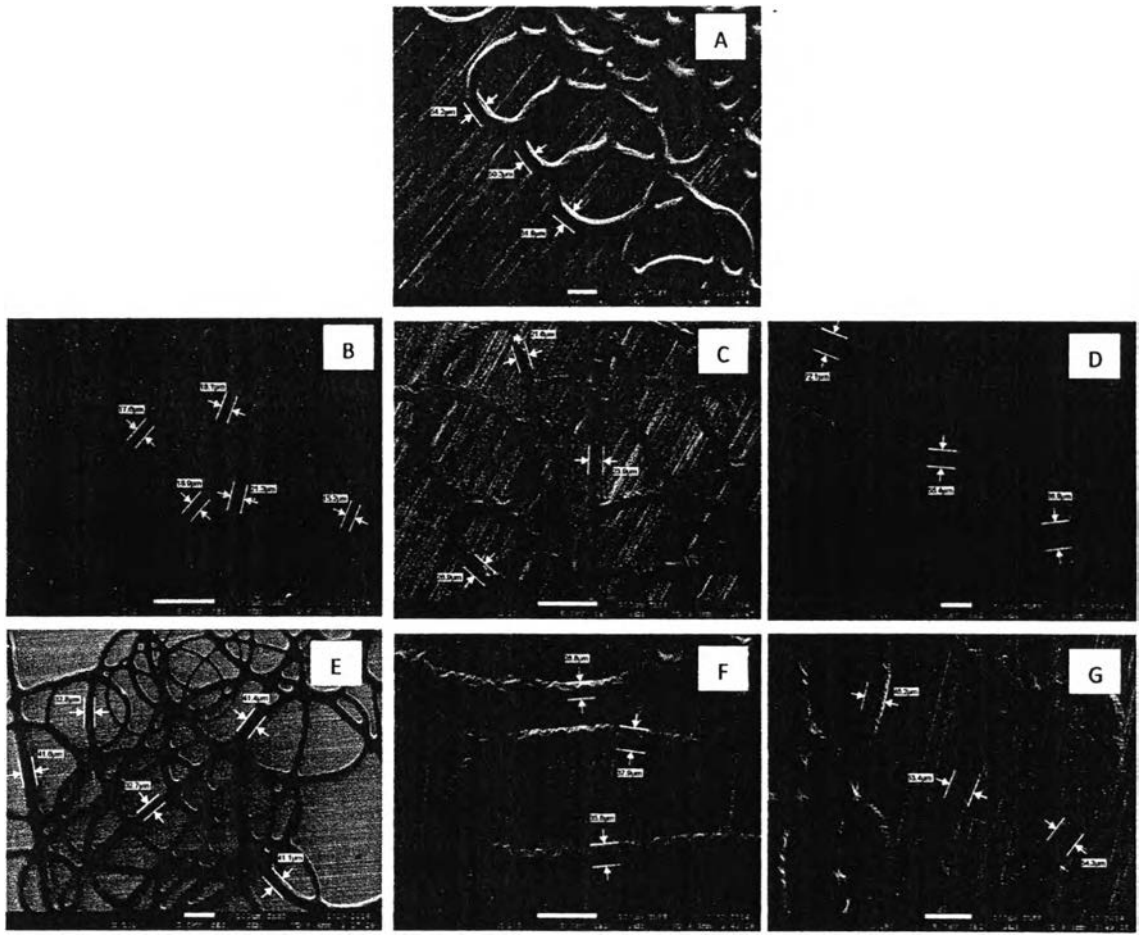

Figure 4.1 SEM images of electrospun fibers at 15 cm of distance and 18 kV of voltage generator : A) PVAc B) PF1.25 C) PFO1.25-5 D) PFO1.25-10 E) PF2.5 F) PFO2.5-5 and G) PFO2.5-10.

Abbreviations PVAc, Pristine PVAc ; PF1.25, PVAc with 1.25 mg/L of CPF ; PFO1.25-5, PVAc with 1.25 mg/mL of CPF and 5%v/v of coconut oil ; PFO1.25-10, PVAc with 1.25 mg/mL of CPF and 10%v/v of coconut oil ; PF2.5, PVAc with 1.25 mg/mL of CPF ; PFO2.5-5, PVAc with 1.25 mg/mL of CPF and 5%v/v of coconut oil ; PFO2.5-10, PVAc with 1.25 mg/mL of CPF and 10%v/v of coconut oil.

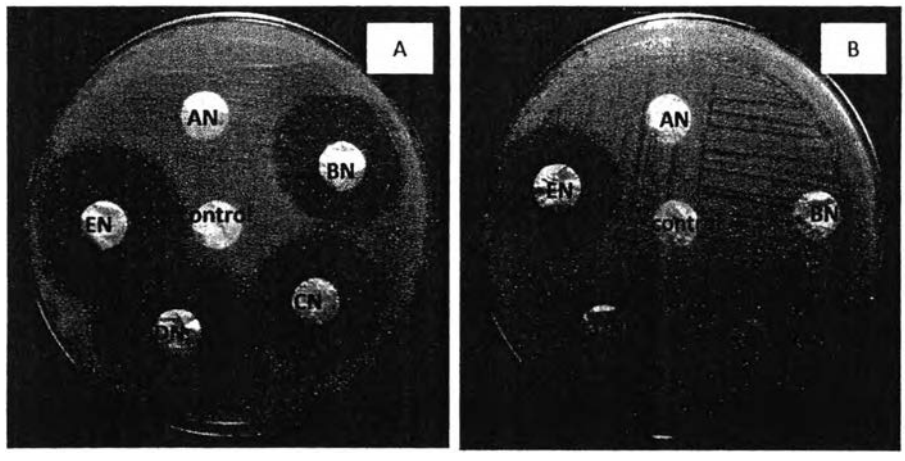

Figure 4.2 Bactericidal activity of pristine PVAc and PVAc with different CPF content electrospun fibers on PU film as mats with A) Gram-negative, *E.coli* and B) Gram-positive, *S.aureus*.

Abbreviations Control, PU film; AN, PVAc; BN, PVAc loaded 0.31 mg/mL of CPF, CN, PVAc loaded 0.625 mg/mL of CPF; DN, PVAc loaded 1.25 mg/mL of CPF; EN, PVAc loaded 2.5 mg/mL of CPF.

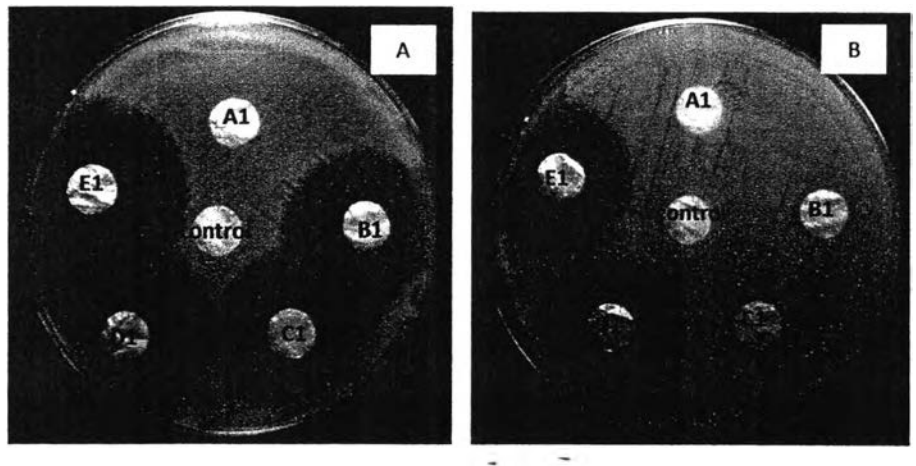

Figure 4.3 Bactericidal activity of pristine PVAc and PVAc with different CPF and 10% v/v of coconut oil content electrospun fibers on PU film as mats with A) Gram-negative, *E.coli* and B) Gram-positive, *S.aureus*.

Abbreviations Control, PU film; A1, PVAc with 10%v/v of oil; B1, PVAc loaded 0.31 mg/mL of CPF and 10%v/v of oil, C1, PVAc loaded 0.625 mg/mL of CPF and 10%v/v of oil; D1, PVAc loaded 1.25 mg/mL of CPF and 10%v/v of oil; E1, PVAc loaded 2.5 mg/mL of CPF and 10%v/v of oil.

The amount of CPF at 1.25 mg/mL could be the minimum concentration for use in order to develop a new dressing. Thus, this content is properly considered to modify the new medical applications. However, the increase of oil content caused some troubles. It can affect to the diameter distribution of electrospun fibers as shown in Figure 4.1. The oil volume of 5%v/v and 10%v/v of oil are inquired for verifying. The bactericidal activity of zone showed obviously the perfect result both microorganisms as shown in Figure 4.4.

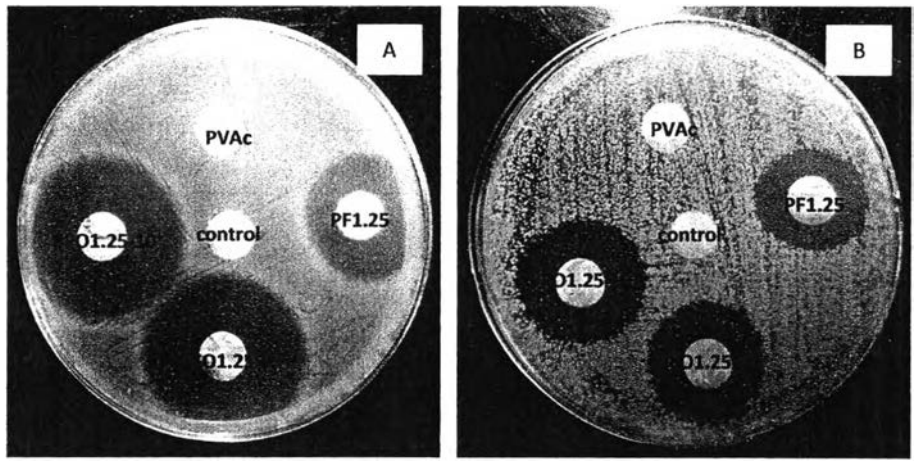

Figure 4.4 Bactericidal activity of pristine PVAc and PVAc with different CPF and coconut oil content of electrospun fibers on PU film as mats with A) Gram-negative, *E.coli* and B) Gram-positive, *S.aureus*.

Abbreviation Control, PU film; PVAc, Pristine PVAc ; PF1.25, PVAc with 1.25 mg/mL of CPF ; PFO1.25-5, PVAc with 1.25 mg/mL of CPF and 5%v/v of coconut oil ; PFO1.25-10, PVAc with 1.25 mg/mL of CPF and 10%v/v of coconut oil.

Table 4.2 Antibacterial activity of the mats against tested bacterial strains

Microorganisms	Clear Zone (mm)[a]				
	control	PVAc	PF1.25	PFO1.25-5	PFO1.25-10
E.coli	NG [b]	NG	8.2±0.3	11.3±0.6	10.1±0.4
S.aureus	NG	NG	6.7±0.5	6.7±0.5	7.6±0.5

[a] Mean ± Standard deviation (n=3)

[b] NG indicate no antibacterial effect on visible growth

The inhibition zone of the materials shown in Table 4.2 noted that the increasing volume of oil tend to accrue the clear zone outstandingly as displayed in Figure 4, especially in *E.coli*, from 8.2 mm to 11.3 mm and 10.1 mm for PFO1.25-5 and PFO1.25-10 mats, respectively. Conversely, the inhibited zone for *S.aureus* is not much different when the coconut oil is multiplied. Therefore, the mats tested in *E.coli* have higher effective than *S.aureus* as the previous reasons.

4.3.2 The Bacterial Reduction Studies

The efficacy of dressing can be assessed by using time-kill kinetics to document antimicrobial activity, and the ability to kill bacteria and prevent re-growth. All sample materials were evaluated using time-kill kinetics. Following standard methodology Gram-positive and Gram-negative bacteria were exposed to each mat and also control at 1 and 24 hours. Each assay was repeated three times and results averaged. *In vitro* reduction studies using documented Gram-positive and Gram-negative pathogens found that both bacteria had different effectiveness in reducing microbial counts. In term of *E.coli* as shown in Figure 4.5, at 1 hour, PFO1.25-5 and PFO1.25-10 can decrease the surviving bacteria from 3.10×10^5 CFUs/mL of control to 1.60×10^5 CFUs/mL and 1.60×10^8 CFUs/mL, respectively. These are equal to 48.39% and 41.93% of percent reduction in PFO1.25-5 and PFO1.25-10, respectively. At 24 hours later, the antibacterial efficiency of dressing is increasing by declining the bacteria from 1.09×10^7 CFUs/mL of control sample to

2.00×10^5 CFUs/mL for both PFO1.25-5 and PFO1.25-10. Resulting in percent reduction is 98.86% both PFO1.25-5 and PFO1.25-10.

On the other hands, the bacterial inhibiting efficiency of dressing against *S.aureus* is obviously lower than *E.coli*. At 1 hour as shown in Figure 4.6, the reducing bacteria is from 4.30×10^5 CFUs/mL of control sample to 1.75×10^5 and 1.25×10^5 CFUs/mL which are equivalent to 59% and 70.93% of percent reduction of PFO1.25-5 and PFO1.25-10, respectively. Furthermore, the bacterial reduction after 24 hours is decreased when compared with the control sample which the cell viability of *S.aureus* is declined from 5.00×10^{11} CFUs/mL to 3.30×10^{11} and 3.65×10^{11} CFUs/mL indicating the percent reduction is 34.00% and 27.00% of PFO1.25-5 and PFO1.25-10, respectively. The results pointed that the survival bacteria at 24 hour tends to increase because they can fully flourish in the environmental of culturing in carrier even if they are in the treated carriers. Therefore, the percentage of bacterial reduction is less.

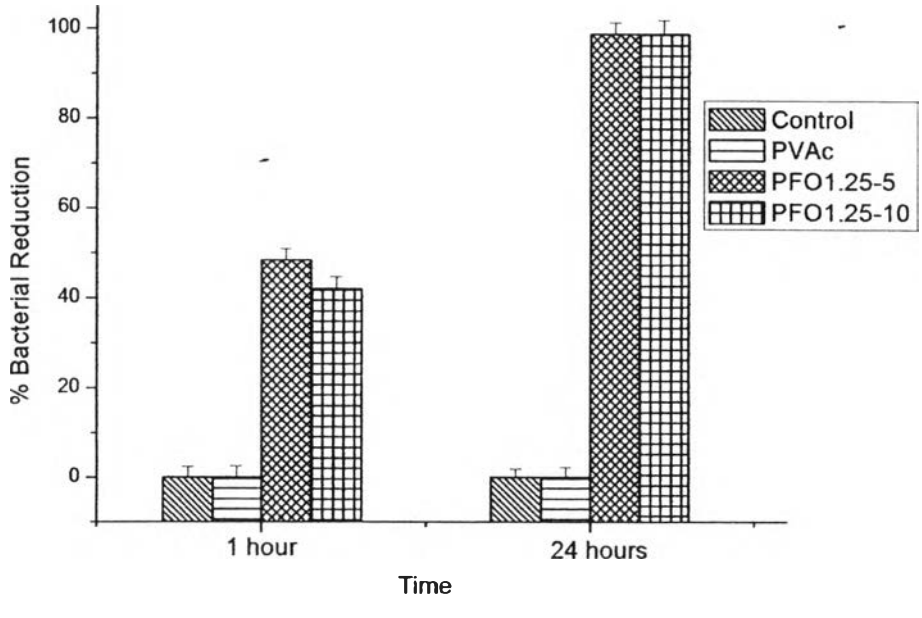

Figure 4.5 The percentage of bacterial reduction of *E.coli* against mats dressings over 1 and 24 h period *in vitro*.

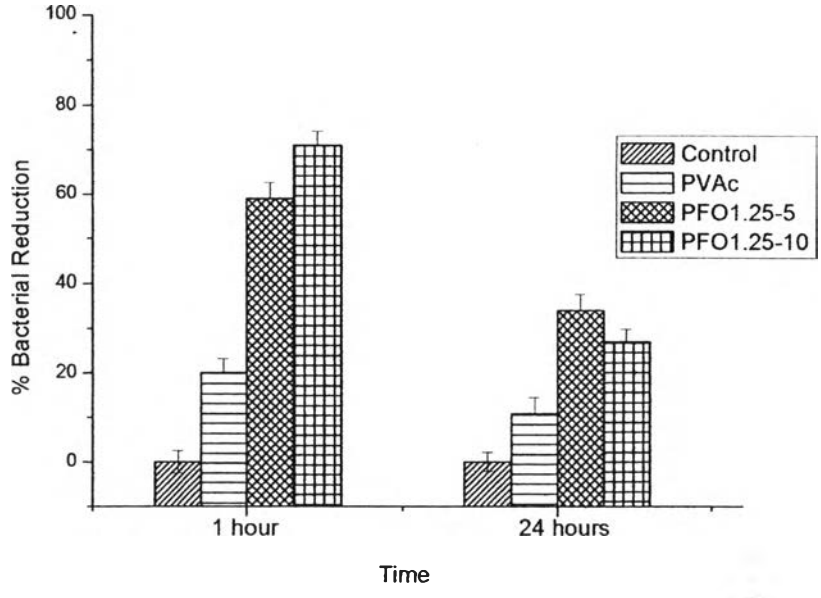

Figure 4.6 The percentage of bacterial reduction of *S.aureus* against mats dressings over 1 and 24 h period *in vitro*.

4.4 Cytotoxicity Test

In vitro cell culturing, the sample mats were tested with human fibroblast and mouse fibroblast cells (L929) by following indirection cytotoxicity evaluation (Li *et al.*, 2011). This method determined how the viability of living cells was after they contacted with extracted media released from the mats by varying time into 1 and 3 days according to using in term of the real medical applications. In term of L929 cells, %cell viability is acceptable for all materials because a viability percentage more than 80%, signifying that all tested materials did not significantly be toxic over the cells all duration test. Observing for almost the sample mats at 1 day, the percentage of survival cells is lower than in 3 days, especially in PFO1.25. It meant that the content of released CPF in the mats was obviously a little toxic for L929 cells. Moreover, the efficiency of coconut oil was also observed. The cell viability can reach to 94.69% and 93.12% for PFO1.25-5 and PFO1.25-10, respectively. Fortunately, the reepithelialization of cells after 3 days tended to augment for almost mats which were the good result for applying to the medical applications without concerning about the cytotoxicity.

Conversely, the cell viability of human fibroblast cells were significantly enhancing when they contacted with the media released from mats. All mats were successfully overcame the cytotoxicity standard, more than 80% of cell viability.

Almost of them can be more than 100% of control at 1 day and they also were continued to reepithelialize cells after then. These results showed that the mats can definitely enhance or promote the wound healing as the above results.

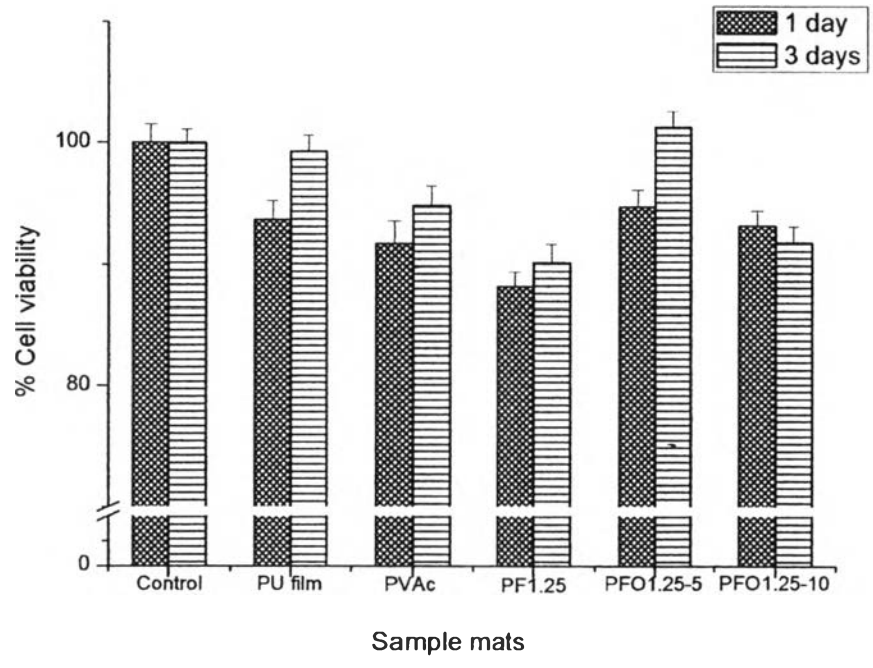

Figure 4.7 Indirect cytotoxicity evaluation of mouse fibroblast cells (L929) cultured on five mats, PU film, PVAc, PFO1.25, PFO1.25-5, and PFO1.25-10.

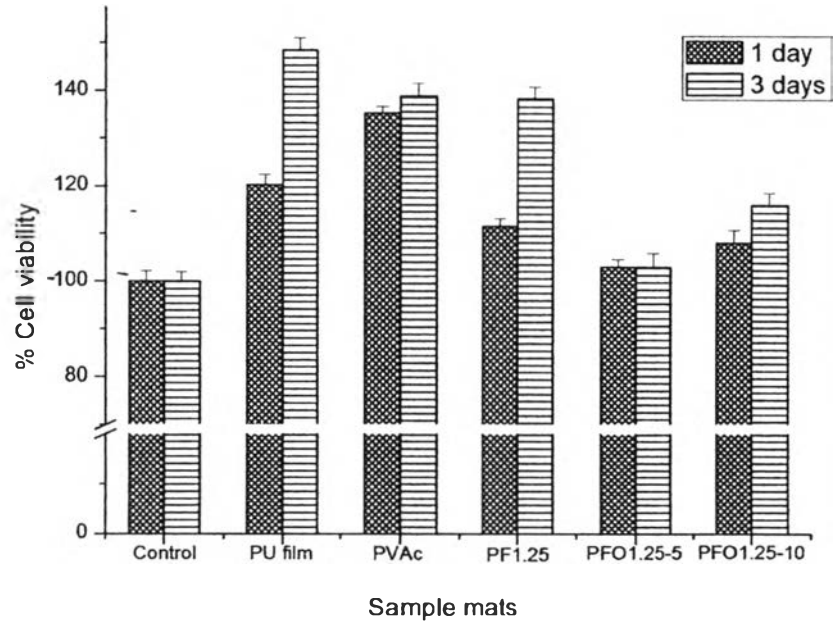

Figure 4.8 Indirect cytotoxicity evaluation of human fibroblast cells cultured on five mats, PU film, PVAc, PF1.25, PFO1.25-5, and PFO1.25-10.

4.5 Characterization and Testing

4.5.1 *In Vitro* Drug Release Study

According to Figure 4.9 and 4.10, the release profile of all dressing mats tended to be the same release. It has been found that the one of factors contributing to drug release in release systems is the behavior of the matrix loaded with the CPF in the release medium. All dressings showed a very quick on initial release during before 6 hours because the distribution diameter of electrospun fibers typically had the drug content on shells or circular fibers more than the core of electrospun fibers. Therefore, it causes a quick burst release in initial time. In addition, in accordance with the lowest degree of swelling along with the dissolution of the PVAc mats, the drug release will be steady after 6 hours due to its high stability in aqueous. The ability of all mats in releasing drug will have been slower and turned to be constant studied previously (Jannesari *et al.*, 2011) . Moreover, the release profile in different solution had been observed. The drug release in acetate buffer (pH 5.5), *in vitro* human blood pH, the percentage of cumulative drug release can reach to 38.48%, 34% and 29.92% of PF1.25, PFO1.25-5, and PFO1.25-10, respectively. In contrast, in PBS buffer (pH 7.4) *in vitro* human skin pH, 26.56%, 23.36% and 19.70% were the increasing content measured. Due to the properties and structure of CPF as weak acid, the tendency to release the drug would be multiply when the mats stayed in acid state more than in basic state. Thus, the concentration of release medium in acetate buffer, weak acid solution, could also be more.

4.5.2 Contact Angle of Films Made by PVAc Solutions with Various Content CPF and Coconut oil

Figure 4.11 showed the contact angles of PVAc used for producing electrospun fibers on PU films with various amount of CPF and coconut oil. In PU film, the contact angle is high because the mats formed the hydrophobic property which PU surface will imitates the microstructure and super-hydrophobicity of lotus leaves in that cannot let the hydrate structure pass through. (Zhao *et al.*, 2006). The contact angle of mats films then decreased with applying the content of CPF and coconut oil. This decrease was due to a greater amount of chemicals found in the

hydrophilic group (-OH) structured from CPF in the molecular chains of PVAc which can be formed properly in hydrate structure (Turel *et al.*, 1997). Some found in the hydrophobic group (-COOR) from the coconut oil due to the triglyceride structure of oil. This study showed that the mats were still hydrophobic as the size of contact angle between the material and water was big as the PU film as showed in Figure 4.12.

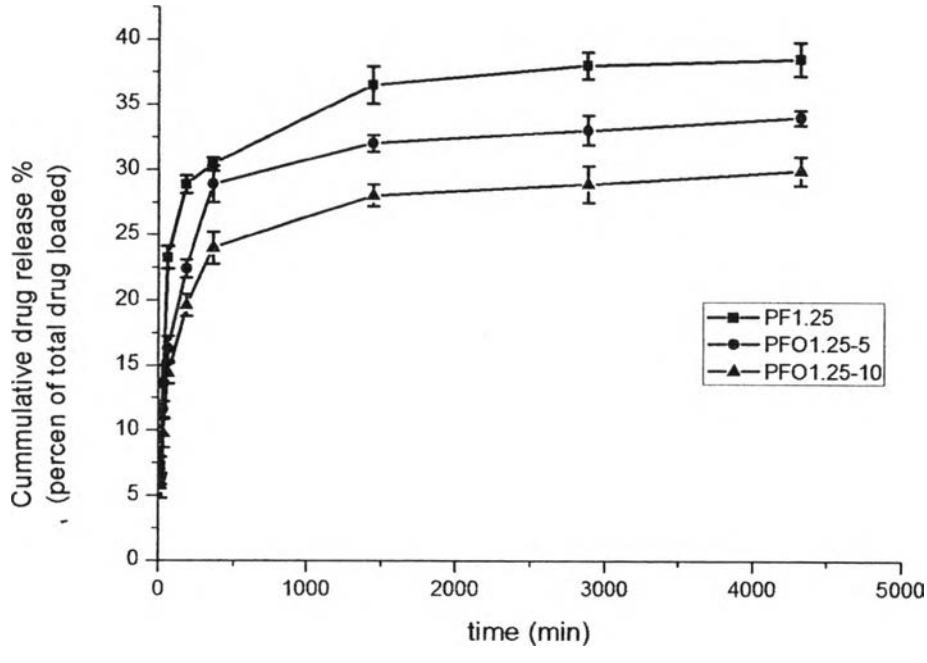

Figure 4.9 Effect of electrospun on the release profile of CPF from medicated electrospun mats containing 1.25 mg/mL of CPF in acetate buffer (pH 5.5).

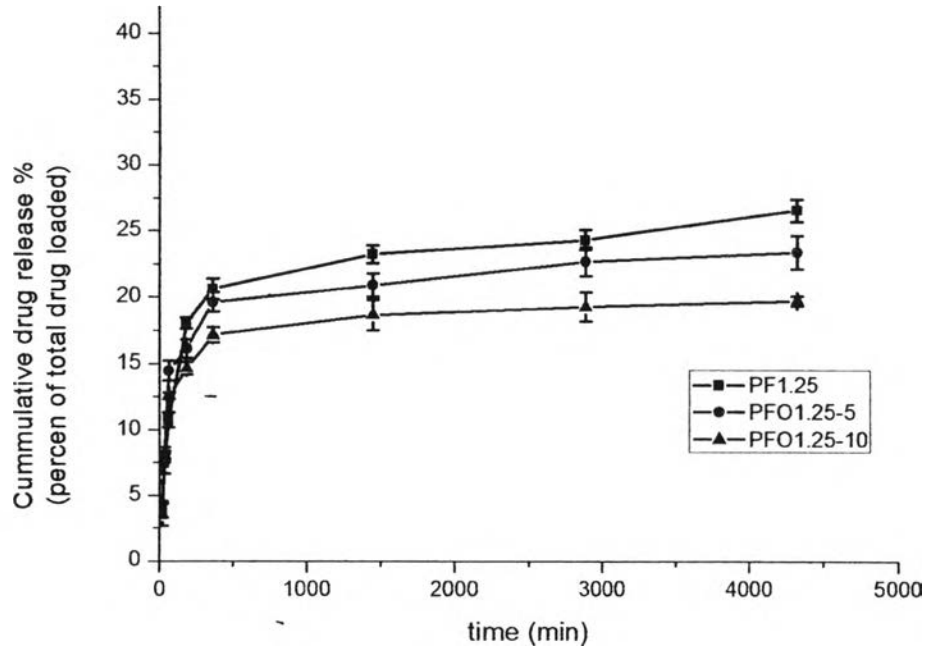

Figure 4.10 Effect of electrospun on the release profile of CPF from medicated electrospun mats containing 1.25 mg/mL of CPF in PBS (pH 7.4).

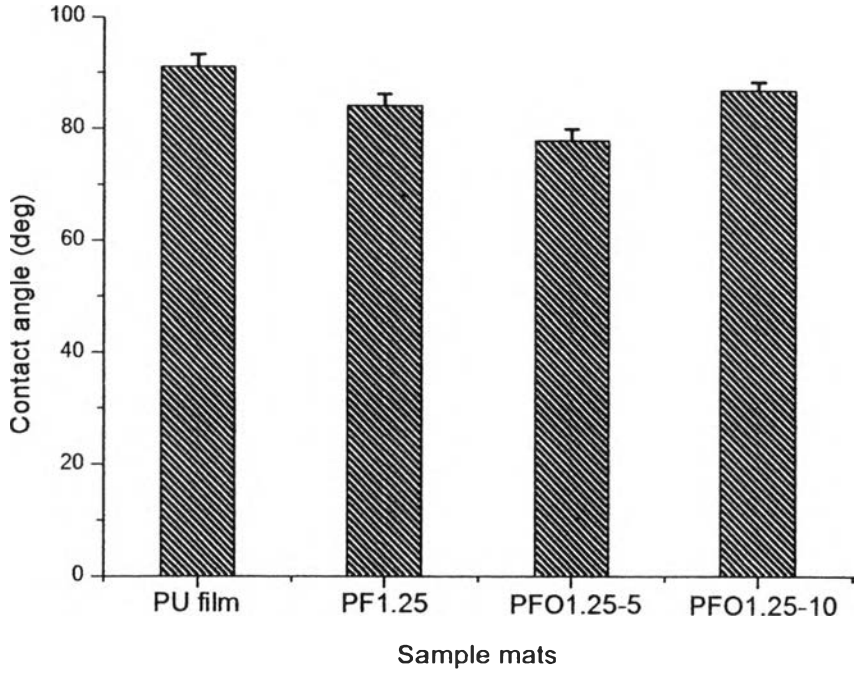

Figure 4.11 Contact angles as related to various CPF and coconut oil volume ratios of the resulting films.

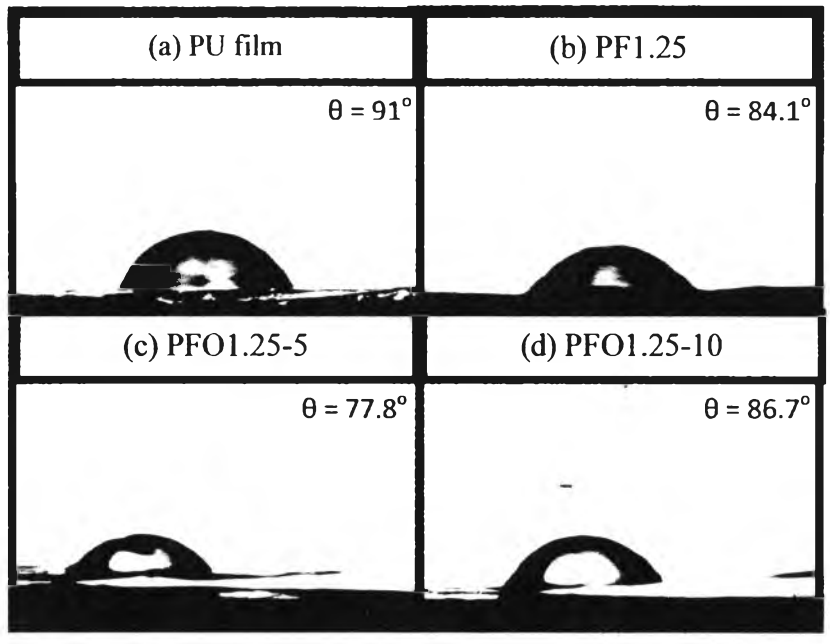

Figure 4.12 Photograph of water droplet on the mats (a) PU film (b) PF1.25, (c) PFO1.25-5, and (d) PFO1.25-10.

4.5.3 FTIR Spectroscopy

The electrospun fiber of PFO1.25-5 and PFO1.25-10 was measured comparing with pristine PVAc, CPF and coconut oil by FTIR spectroscopy as shown in Figure 4.13. Both of them showed clearly peaks about 2,800 cm^{-1} ($-CH_3$,$-CH_2$ stretching), medium peak about 3,000-2,500 cm^{-1} (-OH stretching), and strong peak at 1,700 cm^{-1} (-CO stretching). Comparing with main peaks of CPF, about 3,500-3,450 cm^{-1} are responsible for O-H stretching vibration, about 3,000-2,950 cm^{-1} shows =CH and Ar-H of vibration, and 1,650-1,600 cm^{-1} is N-H bending vibration of quinolines. Observing the spectrum, main peaks of CPF did not show clearly their peak in spectrum of both mats even if they definitely showed the antimicrobial activity. Owing to the high content of PVAc, the peaks of them were totally overlapped. Therefore, all characteristic of the mats showed distinctly high transmittance of PVAc. These noted that the mats had the prominent property because of their spectrum.

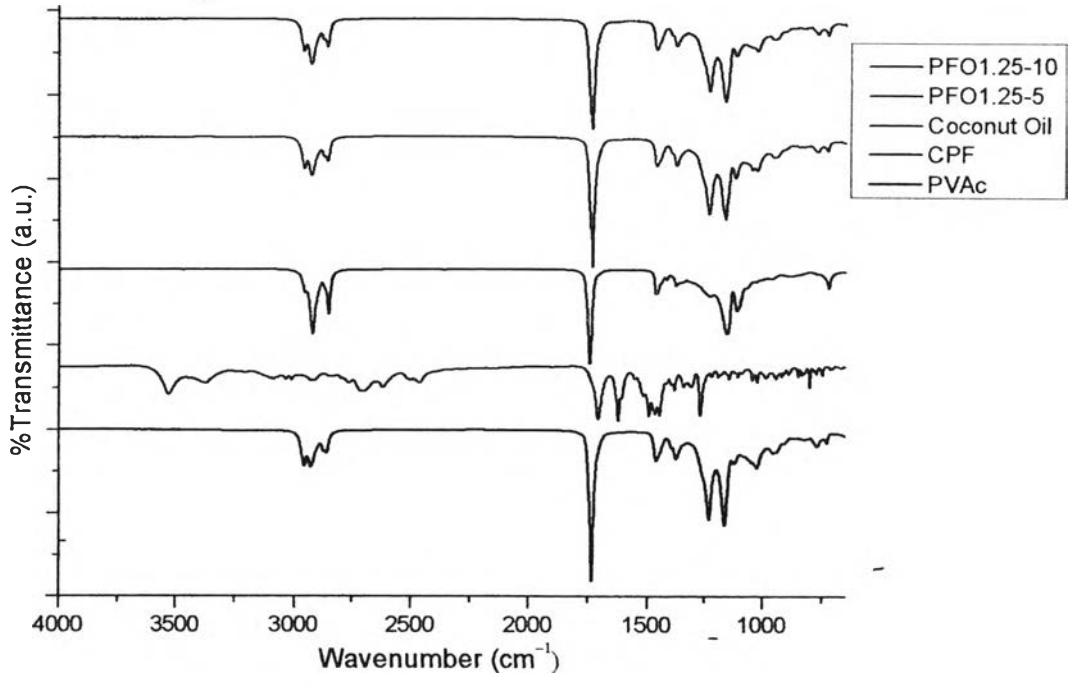

Figure 4.13 FT-IR spectrum of pristine PVAc, CPF and coconut oil and PFO1.25-5 and PFO1.25-10 comparing with pure CPF and Coconut oil.