CHAPTER 4

Results and discussion

4.1 Propolis extraction

4.1.1 Percent yield of propolis extraction

Previous studies have shown that ultrasonic extraction is the most efficient extraction method to obtain propolis [72]. Advantages of this method are yield, short extraction time and high selectivity [76]. Each extraction of propolis using the ultrasonic technique followed by a lyophilization process renders dry propolis extract that can be calculated as percentage yield as shown in Table 4.1

Table 4.1 Percent yield of propolis extract from each extraction

Extraction Number	Weight of propolis Before extraction (g)	Weight of propolis After extraction (g)	Yield (%)
1	30.00	7.93	26.43
2	30.00	9.93	33.10
3	30.00	8.59	28.63
4	30.00	9.20	30.67
		8.91 ± 0.85	29.71 ± 2.85

4.1.2 Total phenolic content in propolis extract

According to the literature [62,77], the majority of compounds identified in propolis are polyphenols. Due to the proven ability of polyphenols to scavenge free radicals, inhibit specific enzymes, stimulate some hormones and neurotransmitters including antimicrobial and antioxidant activities, these are considered to be the main pharmacological compounds in propolis [64]. To determine phenolic compounds to analyze propolis extract before using it as active ingredient, the Folin-Ciocalteu method was used. This method expresses the total phenolic content per mg of Gallic acid equivalents per g of propolis (GAEs), calculated by making use of the calibration curve ($y = 10.496x + 0.0671$, $R^2 = 0.999$) of a Gallic acid standard solution, as shown in appendix A. The results of total phenolic content in propolis extract from each extraction (n = 3) are shown in Table 4.2

Table 4.2 Concentration (mg/g) of phenolics in propolis extracts from different extractions

Extraction number	Phenolics (mg/g)
1	162.47 ± 3.86
2	161.62 ± 0.74
3	167.10 ± 1.62
4	166.08 ± 0.93
Average ± SD	164.32 ± 2.68

Due to the differences in their chemical compositions, the biological activities of propolis from different areas of origin are also different. Kumazawa et al. [78] studied different collection sites of propolis: Argentina, Australia, Bulgaria, Chile, China, Hungary, New Zealand, South Africa, Thailand, Ukraine, Uruguay, United States, Uzbekistan and Brazil.

Ethanol extracts of propolis were prepared. The total polyphenol and flavonoid contents of various propolis samples were tested and evaluated on antioxidant activities. The results showed that propolis from various countries were different in terms of total polyphenol and flavonoid influence in antioxidant activities. The range of total polyphenol in the study was between 31.2 – 299 mg/g of ethanol extracts of propolis from various sources. Silva et al. [79] compared the efficacy of three extract methods: hydro-alcoholic, methanolic and aqueous; he examined polyphenol content and antimicrobial activity from propolis harvested in Portugal. The hydro-alcoholic was found to be the best solvent to extract the content of phenolics and flavonoids compounds from propolis. Propolis from different places had different concentrations of polyphenols. All propolis extracts in this study can be used in antimicrobial activities but the effect depends on the origin of the extract and the microorganisms.

Based on the Silva study, the hydro-alcoholic method was chosen to extract propolis and study its total phenolic content. The results showed that the total phenolic content from each extraction was not different. Brazilian propolis was chosen as active ingredient in this study which had an average concentration of phenolics of 164.32 ± 2.68 mg/g.

4.2 Preparation of polymer - based fibers by using the electrospinning technique

4.2.1 Preparation of polymer based electrospun fibers

1) Preparation of polyvinyl pyrrolidone electrospun fibers

High molecular weight PVP (PVP K90) and low molecular weight PVP (PVP K30) were prepared as electrospinning solutions at various concentrations to screen the morphology of the fibers. The results are shown in Table 4.3 and Figure 4.1 for PVP K90 polymer solution, while Table 4.4 and Figure 4.2 show results for PVP K30 polymer solution.

Table 4.3 Experimental data of PVP K90 polymer solutions for the electrospinning process and fiber diameters obtained.

Concentration of PVP K90 polymer solution (% w/v)	Solvent	Formation	Diameter of fibers (μm)
4%	Ethanol	Bead ++	-
6%	Ethanol	Bead +	-
8%	Ethanol	Uniform fibers	0.43 ± 0.09
10%	Ethanol	Uniform fibers	0.64 ± 0.16

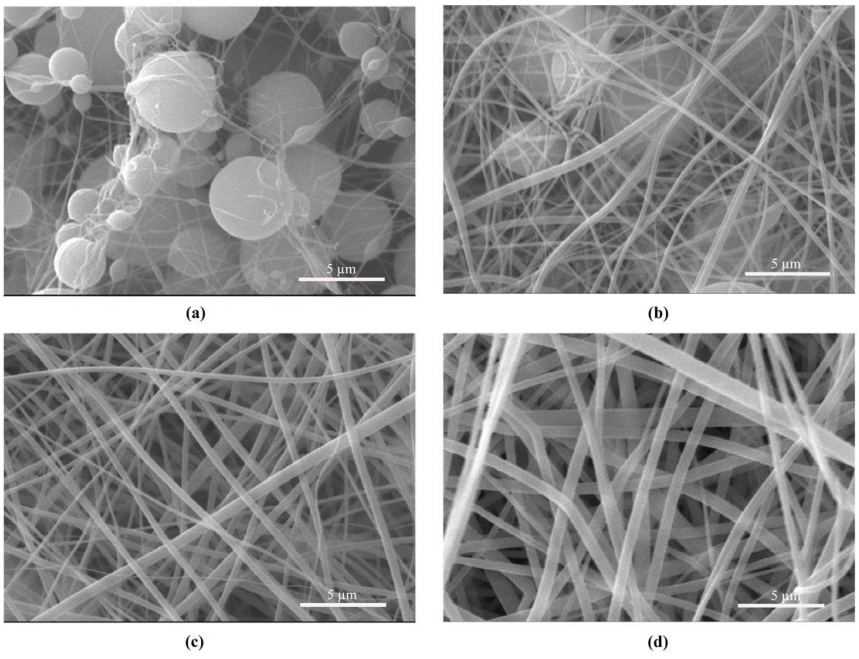


Figure 4.1 SEM photographs of PVP K90 electrospun fibers at (a) 4% (w/v), (b) 6% (w/v), (c) 8% (w/v) and (d) 10% (w/v).

Table 4.4 Experimental data of PVP K30 polymer solutions for the electrospinning process and fiber diameters obtained.

Concentration of PVP K30 polymer solution (% w/v)	Solvent	Formation	Diameter of fibers (μm)
30%	Ethanol	Bead ++	-
35%	Ethanol	Bead +	-
40%	Ethanol	Uniform fibers	2.18 ± 0.35

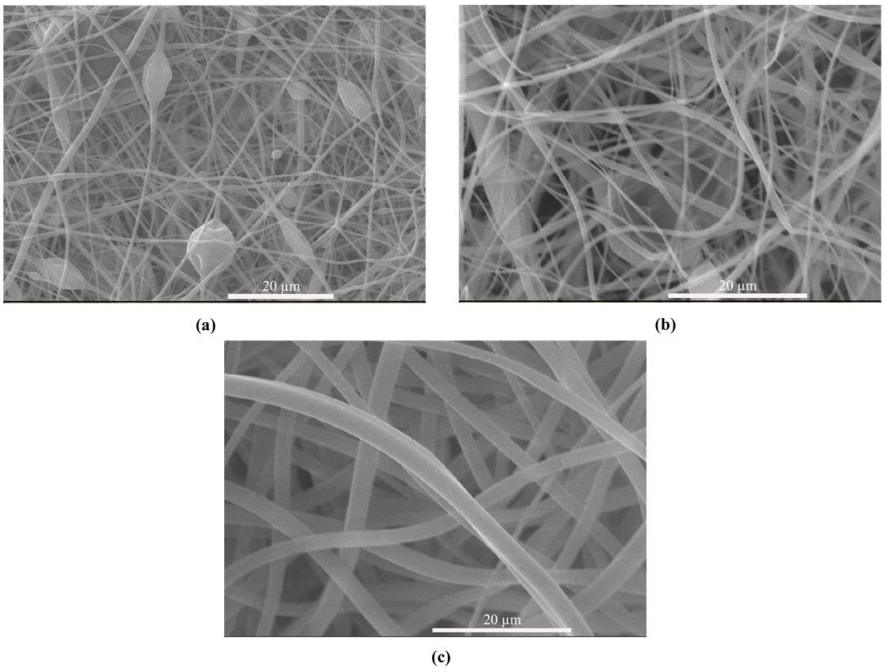


Figure 4.2 SEM photographs of PVP K30 electrospun fibers at (a) 30% (w/v), (b) 35% (w/v) and (c) 40% (w/v).

2) Preparation of polyvinyl alcohol electrospun fibers

High molecular weight PVA (MW 85,000 – 146,000) and low molecular weight PVA (MW 47,000) were prepared as electrospinning solutions at various concentrations to observe the morphology of the fibers. The results are shown in Table 4.5 and Figure 4.3 for PVA (MW 85,000 – 146,000) polymer solution, while Table 4.6 and Figure 4.4 show the results for the PVA (MW 47,000) polymer solution.

Table 4.5 Experimental data of the PVA (MW 85,000 – 146,000) polymer solutions for the electrospinning process, and fiber diameters obtained.

Concentration of PVA (MW 85,000 – 146,000) polymer solution (% w/v)	Solvent	Formation	Diameter of fibers (μm)
6%	Water	Bead ++	-
8%	Water	Bead +	-
10%	Water	Uniform fibers	0.18 ± 0.03

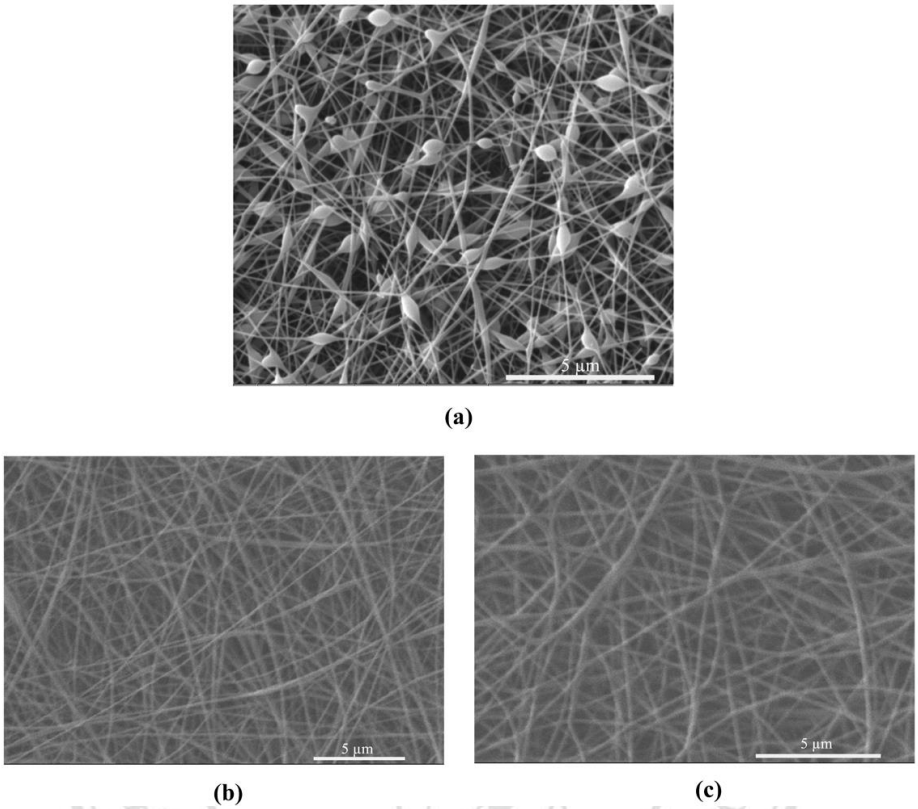


Figure 4.3 SEM photographs of PVA (MW 85,000 – 146,000) electrospun fibers at (a) 6% (w/v), (b) 8% (w/v) and (c) 10% (w/v).

Table 4.6 Experimental data of PVA (MW 47,000) polymer solutions for the electrospinning process and fiber diameters obtained.

Concentration of PVP K30 polymer solution (% w/v)	Solvent	Formation	Diameter of fibers (μm)
20%	Water	Bead ++	-
25%	Water	Bead +	-
30%	Water	Uniform fibers	0.19 ± 0.05

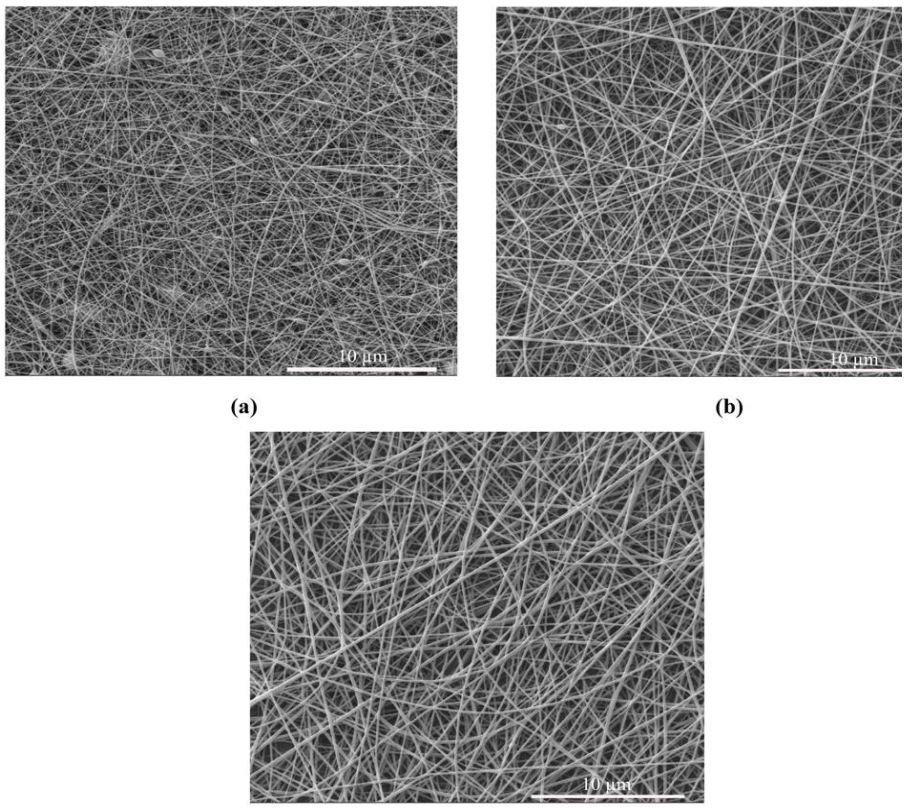


Figure 4.4 SEM photographs of PVA (MW 47,000) electrospun fibers at (a) 20% (w/v), (b) 25% (w/v) and (c) 30% (w/v).

3) Preparation of hydroxylpropyl cellulose electrospun fibers

A solution of 10 % (w/v) – 15% (w/v) of hydroxylpropyl cellulose (MW 370,000) was prepared for the electrospinning process. It was found that using an electric potential ranging from 15 – 25 kV resulted in droplets or a pulpy structure of HPC with no fiber formation at a concentration of 10 % (w/v) HPC. As for 15% (w/v) HPC, electrospun fibers with bead formation were formed.

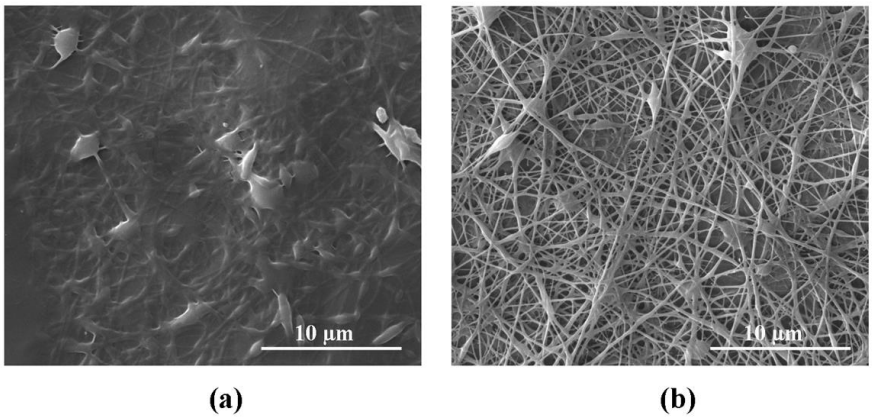


Figure 4.5 SEM photographs of HPC (MW 370,000) electrospun fibers at (a) 10% (w/v), and (b) 15% (w/v).

Molecular weight and concentration of the polymer are critical factors influencing the morphology of electrospun fibers [1,4]. This part of the study investigated suitable concentrations of each water-soluble polymer for the electrospinning process. Different molecular weights of PVP, PVA and HPC at various concentrations were studied. The viscosity of the polymer solution increased when the concentration of polymer solution increased. It was found that morphology and fiber diameters were dependent on the polymer concentration. A sample of PVP K90 4% (w/v) showed much bead formation and when the concentration was increased to 6% (w/v), bead formation decreased with more fibers. Smooth and uniform fibers with diameters in the range of 0.43–0.64 µm

were obtained when the concentration of PVP K90 was 8-10% (w/v). In the case of PVP K30, the concentration of PVP K30 was increased to 40% (w/v) to produce uniform electrospun fibers without bead formation with an average diameter of 2.18 μm. For PVA (MW 85,000-146,000), the results showed that a sample with 6% (w/v) concentration produced electrospun fibers mixed with bead formation. Bead formation decreased with increased polymer concentration. When the spinning concentration of PVA (MW 85,000-146,000) was increased to 10% (w/v), smooth and uniform fibers with an average diameter of around 0.18 μm were obtained. As for low molecular weight PVA (MW 47,000), 30% (w/v) concentration of polymer solution appeared to be the suitable concentration to produce nanospun fibers that were free from bead formation and had an average diameter of 0.19 μm. It was concluded that low concentration polymer solutions resulted in more bead formation than fibers. With increased concentration of the polymer solution, smooth and uniform fibers were formed and the diameter of fibers increased as well, because the high concentration of polymer solution increased the solution viscosity and the chain entanglements were sufficient to stabilize the polymer jet along the distance to the collector [80,81]. For the low molecular weight polymers (PVP K30 and PVA MW 47,000), higher concentrations of polymer solution were used when compared with high molecular weight polymers (PVP K90 and PVA MW 85,000-146,000) to produce good morphology of electrospun fibers. In the case of HPC (MW 370,000), it was found that the formation of HPC electrospun fibers was difficult. According to results obtained in a study of Yan et al. [82] and Francis et al. [83], large particles became attached to the collector instead of uniform fiber formation using the electrospinning technique. HPC molecules have a strong tendency to form bigger particles. When the concentration of HPC reaches critical values, the polymer could entangle in chains and form large particles, which made the electrospinning process more difficult. Blending HPC with another polymer was necessary to improve the structural stability

and the charge carrying capacity of the polymer solution to create fiber formation using the electrospinning technique. A study by Francis et al. [83] showed that blending PEO with HPC results in uniformity and beadless nanofibers with an average diameter 255 ± 65 nm using the electrospinning process.

Based on the above overall results, we choose PVP K90 and PVA (MW 85,000-146,000) as these can lead to uniform production and good morphology of electrospun polymer fibers that are useful for further study. The optimal concentration of the polymer solution for further electrospinning processes was set at 10% (w/v) for both.

4) Effect of mixed solvent on polymer-based electrospun fibers

A suitable solvent is one of the important factors that influence the morphology of electrospun fibers [84,85]. Mixed solvents of ethanol and water in various ratios were used in this study. Small amounts of water were mixed with ethanol to dissolve PVP K90 with a concentration of PVP K90 of 10% (w/v). SEM photographs of PVP K90 nanospun fibers produced at different ratios of ethanol:water (10:0, 8:2) are presented in Figure 4.6(a) and (b). When a decreased ratio of ethanol:water (7:3) was used, a droplet of polymer solution appeared on the aluminum foil instead of the nanospun fibers. The concentration of PVP K90 was increased from 10% (w/v) to 12% (w/v) for the next step, with the same ratio of mixing solution of ethanol and water as solvent. Figure 4.6(c)–(e) shows 12% (w/v) PVP K90 electrospun fibers from the polymer solution in ethanol:water at ratios of 10:0, 8:2 and 7:3 respectively.

In the case of PVA (MW 85,000-146,000), which is soluble in water, small amounts of ethanol were mixed with water for the solving process. Various ratios of water:ethanol were used (10:0, 8:2, 7:3 and 5:5) to dissolve 10% (w/v) PVA (MW 85,000-146,000); the SEM images of the resulting nanofibers are shown in Figure 4.7(a)-(d).

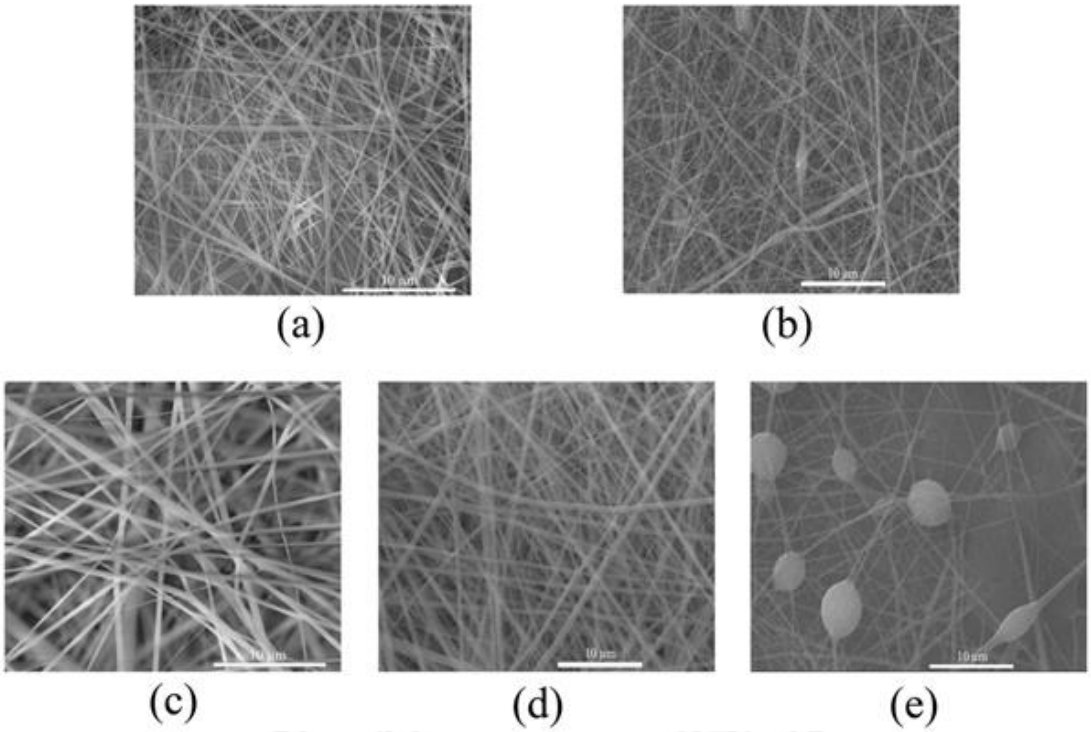


Figure 4.6 SEM photographs of PVP K90 electrospun fibers at a concentration of 10% (w/v) using the following solvents: (a) ethanol, (b) ethanol:water 8:2, and at a concentration of 12% (w/v) PVP K90 in solvents: (c) ethanol, (d) ethanol:water 8:2 and (e) ethanol:water 7:3.

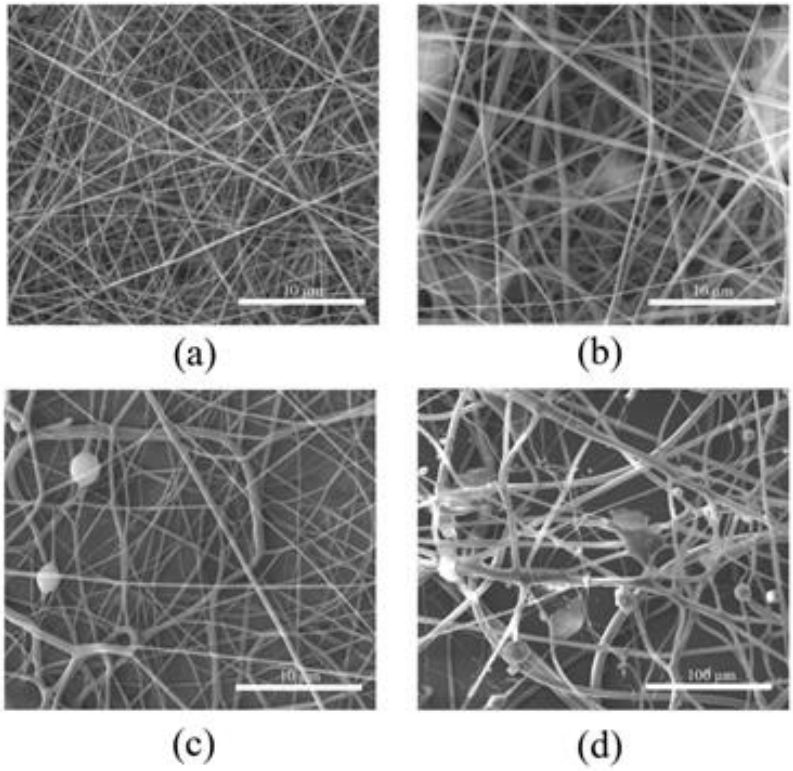


Figure 4.7 SEM photographs of PVA (MW 85,000-146,000) at a concentration of 10% (w/v) using the following solvents: (a) water, (b) water:ethanol 8:2, (c) water:ethanol 7:3, (d) water:ethanol 5:5.

Table 4.7 shows the physical properties of the polymer solutions in terms of conductivity, surface tension, viscosity of electrospinning solution and measured diameters of the electrospun fibers.

Table 4.7 Physical properties of PVP K90 and PVA (MW 85,000-146,000) polymer solutions in different mixed solvents and average electrospun fiber diameters obtained.

Types of solutions	Conductivity (μs/cm)	Surface tension (mN/m)	Viscosity (cP)	Average diameter of fibers (μm)
10% (w/v) PVP K90 in ethanol	11.40	25.45	850.00	0.64 ± 0.16
10% (w/v) PVP K90 in ethanol:water (8:2)	20.06	28.22	< 800.00	0.16 ± 0.08
12% (w/v) PVP K90 in ethanol	11.86	26.06	1163.33	0.68 ± 0.12
12% (w/v) PVP K90 in ethanol:water (8:2)	22.00	28.80	1143.33	0.46 ± 0.09
12% (w/v) PVP K90 in ethanol:water (7:3)	26.60	29.81	1146.67	beads
10% (w/v) PVA in water	1013.33	47.52	1613.33	0.18 ± 0.03
10% (w/v) PVA in water:ethanol (8:2)	581.76	45.72	2056.67	0.35 ± 0.11 + beads
10% (w/v) PVA in water:ethanol (7:3)	453.00	42.30	3283.33	0.35 ± 0.09 + bead
10% (w/v) PVA in water:ethanol (5:5)	357.67	34.74	4290.00	3.65 ± 0.92 + beads

SEM photographs of PVP K90 electrospun fibers showed that adding water (ethanol:water; 8:2) decreased the diameter of the fibers, and the fibers tended to be unstable. Electrospun fibers with some water in the solvent that attached to the collector dissolved in ambient condition after the electrospinning process ended. As for a decreased ratio of ethanol:water (7:3), a droplet of polymer solution on the aluminum foil appeared instead of the nanospun fibers.

When the concentration of PVP K90 was increased from 10% (w/v) to 12% (w/v) with the same ratio by mixing a solution of ethanol, and water used as solvent, the results indicated that the increased concentration of PVP K90 of 12% (w/v) increased the diameter of the fibers, with more stable fiber formation in ambient conditions. Larger amounts of water in this system (ethanol: water; 7:3) resulted in bead formation.

For PVA (MW 85,000-146,000), SEM photographs indicated that adding ethanol to the system influenced the morphology of the fibers. The diameter of fibers increased with increased ethanol in the mixed solvent. At a ratio of ethanol:water of 5:5, nonuniform and very large diameter fibers were formed.

Physical properties of the polymer solutions such as conductivity, surface tension, viscosity and diameters of the electrospun fibers are shown in Table 4.7. It may be concluded that increasing water in ethanol as the mixed solvent for PVP K90 increases the conductivity and surface tension but decreases the viscosity of the electrospinning solvent, which resulted in thinner fiber formation. High amounts of water introduced bead formation (Figure 4.6 (b) and (e)). When the viscosity of the electrospinning decreased, this may have been due to low polymer chain entanglements , thus beads were formed instead of fibers. Water has a low evaporation rate compared with ethanol, so added water will decrease the evaporation rate of the electrospinning solvent. The solvent did not evaporate completely before the polymer jets reached the collector, and this resulted in unstable fibers that dissolved rapidly in an ambient condition after the electrospinning

process. For PVA (MW 85,000-146,000), increased ethanol in the system decreased conductivity and surface tension, but increased viscosity significantly. High viscosity of the electrospinning solution had a greater influence on the morphology of the electrospun fibers. At higher viscosity, it is harder for this electrospinning technique to stretch the polymer jet to create fine fibers, thus the fibers had larger diameters and formed beads (Figure 4.7(c) and 2(d)).

A preliminary study of the dissolution properties of electrospun fibers in water, PVA (MW 85,000-146,000) fibers were found to dissolve within 30 seconds whereas PVP K90 fibers dissolved within 10 seconds. Although PVA (MW 85,000-146,000) produced fibers with good morphology, the dissolution time of fibers was slower than of PVP K90 fibers. This result showed that PVP K90 has strong potential for use as a polymer base in the electrospinning process to produce fast dissolving dosage forms or improve the dissolution of the drugs, as has already been reported in previous studies [30,42]. Thus, the most suitable system for the electrospinning process with added propolis was PVP K90 polymer with pure ethanol as solvent.

Apart from such parameters as the solution concentration, solvent type, the applied voltage, distance between tip and collector, flow rate of the polymer solution, achieving uniformity of the nanospun fibers must be taken into account. Humidity and temperature also play an important role that influences the properties of PVP electrospun fibers. Temperature is the basic parameter to monitor the ambient parameters as it influences the solvent's evaporation and affects solution viscosity [86]. The effect of the humidity on the electrospun fibers depends on the polymer composition. For PVP K90 as used in this study, it was found that it is not possible to obtain good morphology of electrospun fibers at a relative high humidity (RH) of more than 60%. The electrospun fibers fused together as shown in Figure 4.8.

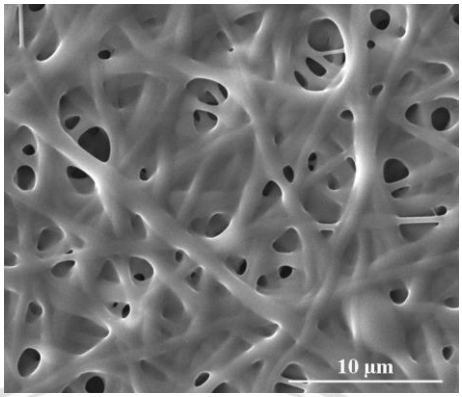


Figure 4.8 SEM photographs of PVP K90 fibers at a concentration of 10% (w/v) using the electrospinning process at 60% RH.

This result is in conformity with a study of Vrieze et al. [86]. The main reason is that the PVP-ethanol solution absorbs ambient water during electrospinning. Incomplete drying causes the fused fibers to deposit on the collector. At higher RH, a transparent film was formed instead of a white PVP electrospun fiber on the collector due to the deposit of a wet electrospun product and its fusion on the collector. There is evidence that lower RH values cause rapid solvent evaporation resulting in thicker nanofibers, whereas higher RH values cause slower solvent evaporation, resulting in thinner nanofibers in various polymers as shown in a study of Pelipenko et al. [87]. Polyvinyl alcohol, and poly ethylene oxide including blended polymers were used to evaluate the effect of RH (4-70%RH) during electrospinning on the morphology of the electrospun fibers. The results showed that RH control during electrospinning can lead to manipulation of the mechanical properties of the polymers. More in particular, temperature and humidity affect the evaporation rate of the solvent and the rigidity of the polymer chain dissolved in the solution. When these two were changed, it was found that the fiber diameters and stability were subject to change as well. In this study the RH was controlled at room temperature under 50 % RH during the electrospinning process.

4.3 Preparation of propolis fast dissolving electrospun fibers

4.3.1 Morphology of propolis-PVP electrospun fibers

Based on the results as referred to above, it may be stated that smooth and uniform fibers were obtained by electrospinning when the concentration of PVP K90 was 8-10%. 2% (w/v) of propolis extract was incorporated into 10% (w/v) and 8% (w/v) PVP K90 and were electrospun. SEM photographs showed that both the 10% (w/v) and 8% (w/v) of PVP K90 polymer solution that incorporated 2% (w/v) propolis, produced smooth and uniform electrospun fibers (Figure 4.9 (a) and 4.9 (b)). The results revealed that the diameter of fibers that were incorporated in the propolis extract increased when compared with PVP K90 electrospun fibers of the same polymer concentration. When the concentration of PVP K90 decreased from 10% (w/v) to 8% (w/v), the average diameter of propolis-PVP electrospun fibers decreased from 0.93 ± 0.20 μm to 0.56 ± 0.16 μm. The results show that with a constant molecular weight of polymer, and as the concentration is decreased, the size of the fibers decreased, affected by the decrease of the viscosity of the electrospinning solution.

To optimize the amount of polymer in electrospun fibers that can produce good morphology, PVP K90 at a concentration of 8% (w/v) was chosen as polymer base. After increasing the propolis extract in the electrospun fibers from 2% (w/v) to 5% (w/v) and 8% (w/v), it was found that a propolis extract of more than 5% (w/v) in a polymer solution created branched electrospinning jets that were ejected from the tip of the needle. Smaller jets were created from the surface of the primary jets. The elongations of the jet and evaporation rate of the solvent from polymer solution had changed and the balance between the electrical forces and surface tension had shifted, causing the shape of the jet to be unstable [13]. Otherwise, some of the branched-jets solidified and attached themselves around the outer diameter of the needle tip. Smooth and uniform fibers could still be formed when propolis extract was added at a concentration of 5% (w/v) into 8% (w/v) PVP K90, as shown in Figure 4.9 (c).

Increased concentrations of propolis extracts from 2% (w/v) to 5% (w/v) in 8% (w/v) PVP K90 increased the size of the fibers from 0.56 ± 0.16 µm to 1.20 ± 0.34 µm.

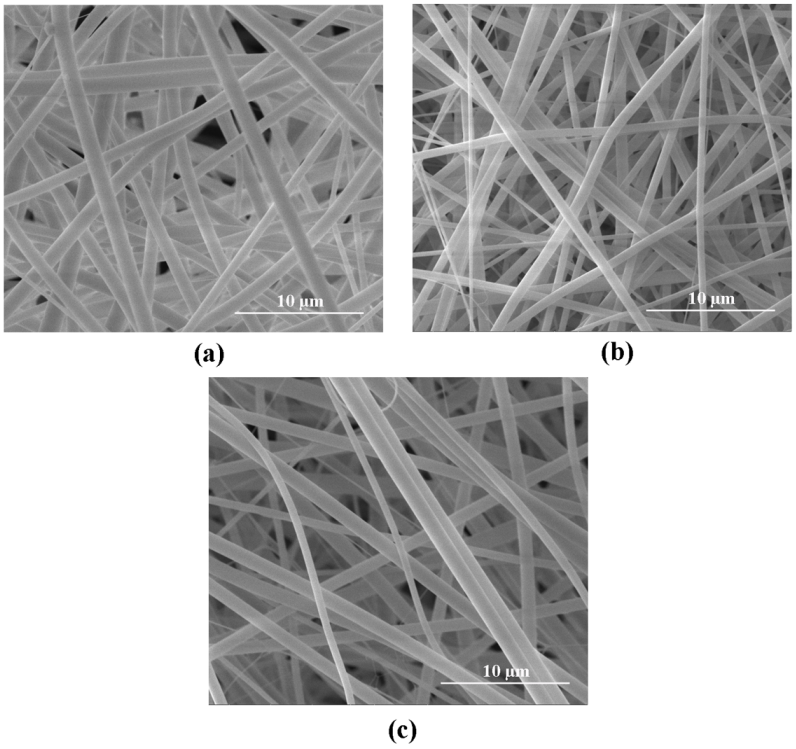


Figure 4.9. SEM photographs of propolis – PVP K90 (a) 10% (w/v) PVP K90 with 2% (w/v) propolis, (b) 8% (w/v) PVP K90 with 2% (w/v) propolis, (c) 8% (w/v) PVP K90 with 5% (w/v) propolis.

To obtain fast dissolving electrospun fibers as oral strips, additives were added in the formulations. All 3 formulations that incorporated propolis as mentioned previously {(10% (w/v) PVP K90 with 2% (w/v) propolis, 8% (w/v) PVP K90 with 2% (w/v) propolis and 8% (w/v) PVP K90 with 5% (w/v) propolis)} added 0.01% (w/v) menthol, 0.005% (w/v) thymol, 0.005% (w/v) methyl salicylate and 0.001% (w/v) eucalyptus oil as flavoring agents. The results revealed that small amounts of additives used as flavoring agent did not influence the electrospun fibers morphology, as shown in Figure 4.10. The average diameter of fibers of 10% (w/v) PVP K90, 2% (w/v) propolis with additives, 8% (w/v) PVP K90, 2% (w/v) propolis with additives and 8% (w/v) PVP K90, 5% (w/v) propolis with additives, were 0.89 ± 0.17 µm, 0.61 ± 0.20 µm and 1.32 ± 0.31 µm, respectively. For further study, 8% (w/v) of PVP K90 was used as a polymer base solution for electrospinning and 5% (w/v) of propolis extract as active ingredient was incorporated.

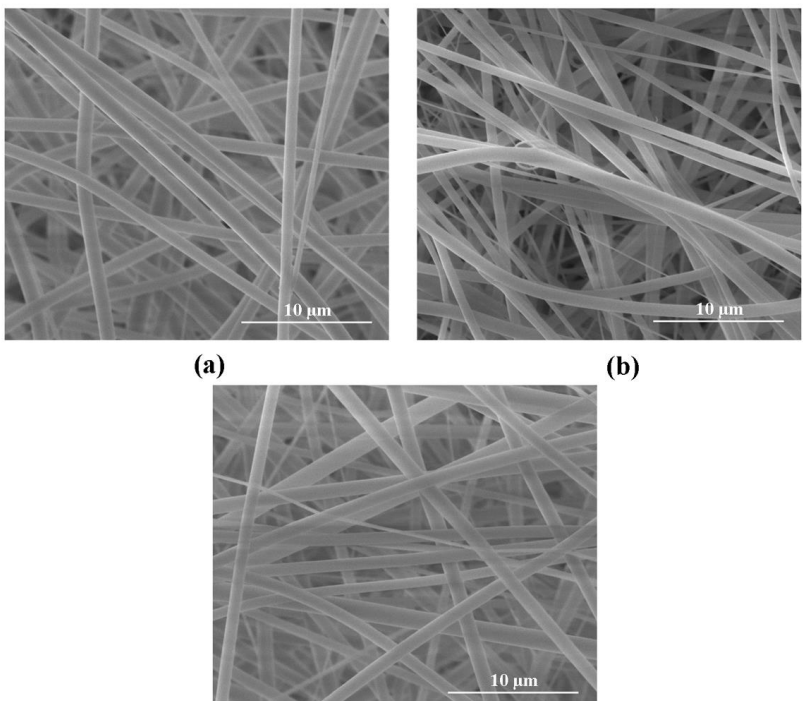
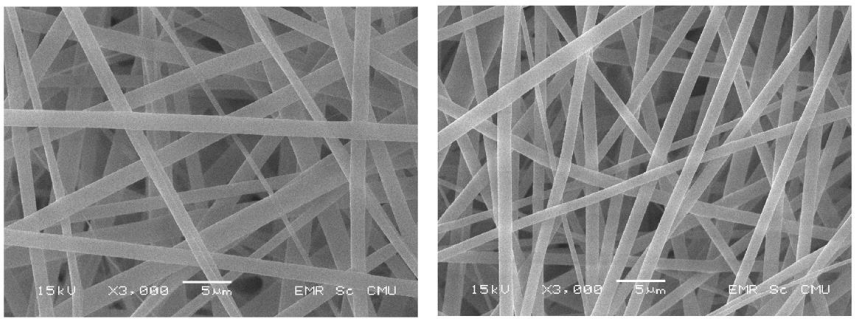
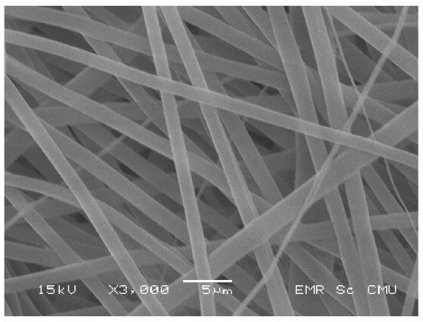


Figure 4.10. SEM photographs of propolis – PVP K90 electrospun fibers (a) 10% (w/v) PVP K90 with 2% (w/v) propolis with additives, (b) 8% (w/v) PVP K90 with 2% (w/v) propolis with additives, (c) 8% (w/v) PVP K90 with 5% (w/v) propolis with additives.

Wettability of the fibers is an important factor for oral fast dissolving film. The required electrospun fibers have to absorb water to make disintegration and dissolution occur in a very short time. PVP is commonly used as a water soluble polymer in oral formulations as it has already proven to be able to enhance the dissolution of poorly soluble solid drugs [88]. It can be spun to fibers by using the electrospinning process to achieve very fine fibers with a large surface area and a high porosity suitable to be used as a polymer base for fast dissolving electrospun fibers. Propolis is mixed with hydrophobic substances that are incorporated in the electrospun. To improve the wettability of propolis-PVP electrospun fibers, Tween 80 is added to the polymer solution as wetting agent. Both 0.5% (w/v) and 1% (w/v) of Tween 80 with 8% (w/v) PVP K90 and 5% (w/v) propolis produce smooth and uniform electrospun fibers as shown in the SEM photographs of Figure 4.11(a) and 4.11 (b). The average diameter of electrospun fibers of 8% (w/v) PVP K90, 5% (w/v) propolis with 0.5% (w/v) Tween 80 and 8% (w/v) PVP K90, 5% (w/v) propolis with 1% (w/v) Tween 80 were 1.59 ± 0.28 µm and 1.28 ± 0.17 µm. Although the concentration of Tween 80 was increased up to 1.5% (w/v), it still can produce nanofibers that attach to the collector, but the electrospun fiber mat seemed to be too sticky to carry out this process properly. 1% (w/v) Tween 80, turned out to be the appropriate concentration to use as wetting agent for propolis-PVP electrospun fibers. Additives were incorporated to formulate fast dissolving electrospun fibers of propolis and the results showed that uniform fibers with good morphology were collected from this formulation as shown in the SEM photographs in Figure 4.11 (c). The average diameter of electrospun fibers of 8% (w/v) PVP K90, 5% (w/v) propolis, 1% (w/v) Tween 80 with additives was 1.43 ± 0.22 µm.



(a) (b)



(c)

Figure 4.11. SEM photographs of 5% (w/v) propolis – 8% (w/v) PVP K90 electrospun fibers with (a) 0.5% (w/v) Tween 80 , (b) 1% (w/v) Tween 80, (c) 1% (w/v) Tween 80 and additives.

In addition, electrospun fibers with chlorhexidine were spun for use as control to study of antimicrobial activities to compare propolis electrospun fibers. 4.8% (w/v) of chlorhexidine was incorporated in an 8% (w/v) PVP K90 solution to fabricate chlorhexidine electrospun fibers using the electrospinning technique, while applying the same process parameters. White electrospun fibers of chlorhexidine electrospun fibers were obtained. Uniform fibers with good morphology with an average diameter of 0.37 ± 0.09 μm are shown in the SEM photograph in Figure 4.12.

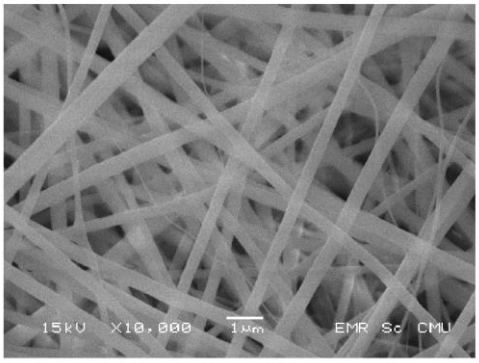


Figure 4.12 SEM photograph of chlorhexidine electrospun fibers.

A summary of the formulations of propolis-PVP K90 electrospun fibers and their average diameters is shown in Table 4.8. The conclusion may be drawn that using a polymer base of PVP K90, an increase of the polymer concentration results in an increase of the fiber diameter due to increased viscosity of the solution, as referred to earlier in this study. Likewise, with the same concentration of PVP K90, an increased amount of propolis results in increasing diameters of the electrospun fibers. Small amounts of additives do not affect the morphology of the electrospun fibers, whereas a small amount of Tween 80 as wetting agent incorporated in the formulations has a slight effect on the diameter increase of the propolis-PVP K90 electrospun fibers.

Formulations with 8% (w/v) of PVP K90 as polymers with 5% (w/v) propolis as active ingredient with or without additives and wetting agent, were studied on the effect of physical properties following electrospinning. The viscosity, conductivity and surface tension of these electrospinning solutions were measured and the results are presented in Table 4.9.

Table 4.8 Summary of the formulations of propolis-PVP K90 electrospun fibers and their average diameters.

Formulations	PVP K90 % (w/v)	Propolis % (w/v)	Additives	Tween 80 % (w/v)	Average diameter (µm)
1	10	-	-	-	0.64 ± 0.16
2	10	2	-	-	0.93 ± 0.20
3	10	2	qs	-	0.89 ± 0.17
4	8	-	-	-	0.43 ± 0.09
5	8	2	-	-	0.56 ± 0.16
6	8	2	qs	-	0.61 ± 0.20
7	8	5	-	-	1.20 ± 0.34
8	8	5	qs	-	1.32 ± 0.31
9	8	5	-	0.5	1.59 ± 0.28
10	8	5	-	1	1.28 ± 0.17
11	8	5	qs	1	1.43 ± 0.22

The results reveal that propolis incorporated in polymer solutions, increases the conductivity and viscosity significantly but does not affect the surface tension of the electrospinning solution. The viscosity plays a role as dominating parameter that affects the size of propolis-PVP electrospun fibers. High viscosity of a propolis-PVP electrospinning solution impact on the diameter increase of the electrospun fibers when compared with PVP based electrospun fibers. Tween 80, the surfactant that reduces the surface tension of the solution, had a synergistic effect on increased viscosity, which resulted in increased diameters of the electrospun fibers.

Table 4.9 Physical properties of various propolis-PVP K90 electrospinning solutions

Formulations	Conductivity (µS/cm)	Surface tension (mN/m)	Viscosity (cP)	Average diameter of fibers (µm)
8% (w/v) PVP K90	6.88 ± 0.01	24.48 ± 0.00	95.00 ± 0.17	0.43 ± 0.09
8% (w/v) PVP K90 +5% (w/v) Propolis	58.00 ± 0.20	24.82 ± 0.29	133.40 ± 0.17	1.20 ± 0.34
8% (w/v) PVP K90 +5% (w/v) Propolis + Additives	58.10 ± 0.75	24.62 ± 0.12	131.70 ± 1.31	1.32 ± 0.31
8% (w/v) PVP K90 +5% (w/v) Propolis + 0.5% Tween 80	49.93 ± 0.12	24.41 ± 0.12	143.60 ± 0.17	1.59 ± 0.28
8% (w/v) PVP K90 +5% (w/v) Propolis + 1% Tween 80	49.83 ± 0.21	24.28 ± 0.00	170.60 ± 0.17	1.28 ± 0.17
8% (w/v) PVP K90 +5% (w/v) Propolis + 1% Tween 80 + Additives	49.43 ± 0.23	24.21 ± 0.12	170.90 ± 0.92	1.43 ± 0.22

4.3.2 Wetting and dissolution time of electrospun fibers

In preliminary testing of wetting and dissolution time of electrospun fibers, it was found that PVP K90 electrospun fibers showed good wettability and had a very high dissolution rate. Hereunder the test results of studying the wetting and dissolution time of PVP K90 electrospun fibers that incorporated propolis as active agent, are presented. Table 4.10, Figure 4.13 and Figure 4.14 refer.

Table 4.10 Wetting and dissolution time of propolis-PVP K90 electrospun fibers

Formulation	Wetting/Dissolution time (sec)
8% (w/v) PVP K90 electrospun fibers	0.72 ± 0.09*
8% (w/v) PVP K90 + chlorhexidine electrospun fibers	0.85 ± 0.06*
8% (w/v) PVP K90 + 5% (w/v) propolis electrospun fibers	45.40 ± 1.51
8% (w/v) PVP K90 + 5% (w/v) propolis + additives electrospun fibers	47.21 ± 2.25
8% (w/v) PVP K90 + 5% (w/v) propolis + 0.5% (w/v) Tween 80 electrospun fibers	5.34 ± 0.94
8% (w/v) PVP K90 + 5% (w/v) propolis + 1% (w/v) Tween 80 electrospun fibers	1.60 ± 0.34
8% (w/v) PVP K90 + 5% (w/v) propolis + 1% (w/v) Tween 80 + additives electrospun fibers	1.67 ± 0.33

* indicated dissolution time of the electrospun fibers

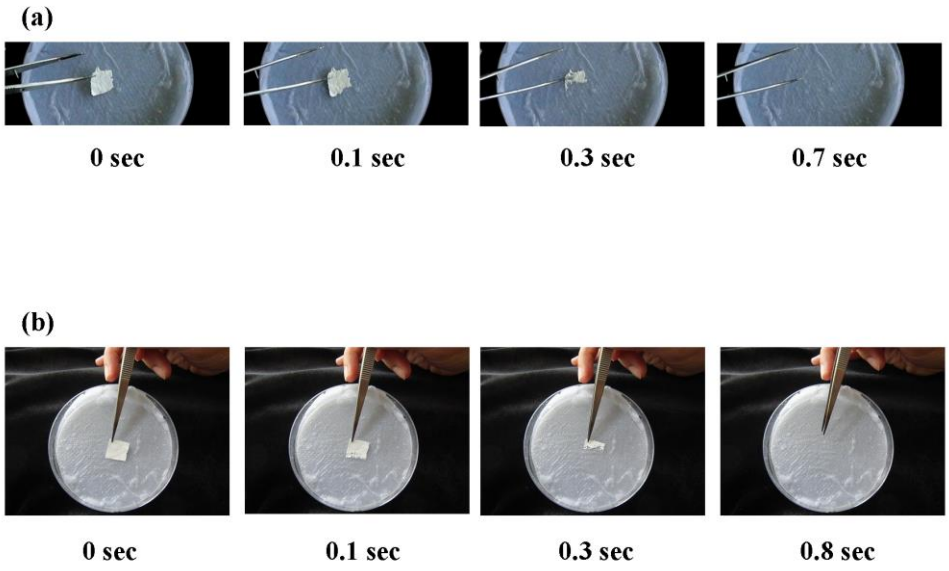


Figure 4.13 The dissolution time of (a) PVP K90 electrospun fibers and (b) chlorhexidine electrospun fibers.

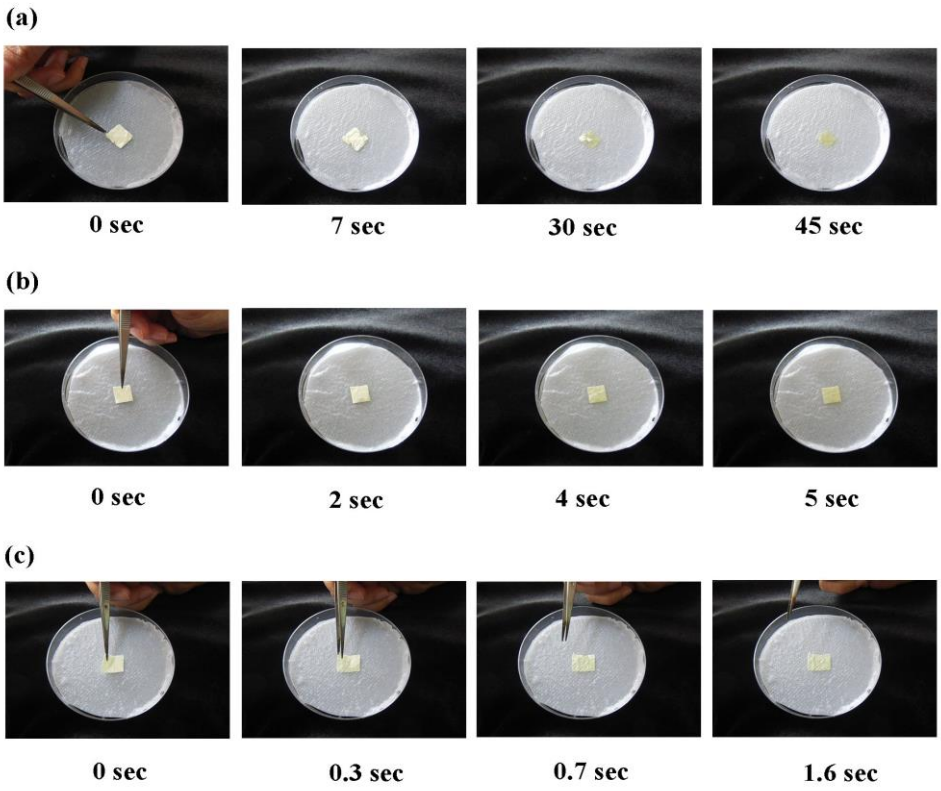


Figure 4.14 The wetting time of propolis-PVP K90 electrospun fibers (a) without Tween 80, and (b) with 0.5% (w/v) Tween 80 and (c) with 1% (w/v) Tween 80.

The results indicated that PVP K90 electrospun fibers showed a very fast dissolution time, i.e. within 1 second. Incorporating chlorhexidine as active ingredients in electrospun fibers did not affect the dissolution property of the electrospun fibers, whereas, incorporating propolis extract into electrospun fibers retarded the wetting and dissolution time of the electrospun fibers. Tween 80 showed improved wettability and enhanced dissolution of the propolis from propolis-PVP electrospun fibers. Propolis-PVP electrospun fibers formed a gel-like structure when the water was absorbed into electrospun fibers before the dissolution process took place next.

Figure 4.15 shows the evidence of releasing the propolis from electrospun fibers. Electrospun fiber mats of 8% (w/v) PVP K90, 5% (w/v) propolis (Figure 4.15(a)) were studied to compare with electrospun fiber mats of 8% (w/v) PVP K90, 5% (w/v) with 0.5% (w/v) Tween 80 (Figure 4.15 (b)) and electrospun fiber mats of 8% (w/v) PVP K90, 5% (w/v) with 1% (w/v) Tween 80 (Figure 4.15(c)). After testing the wetting and dissolution time of electrospun fibers using a digital camera, it was found that a yellow gel-like structure penetrated the wetted paper after some time, showing the release of propolis from the electrospun fibers. Longer distances of the penetration of yellow gel-like structure were observed from the propolis-PVP electrospun fiber mats with 1% (w/v) Tween 80 and compared with the others. It can be concluded that wetting agent Tween 80 improved wettability of the electrospun fibers. The propolis-PVP electrospun fiber mats that contained Tween 80 absorbed water faster and formed a gel like structure. After the water was absorbed into the structure of the electrospun fibers, PVP K90 dissolved rapidly and promoted propolis to be dissolved and released from electrospun fibers.

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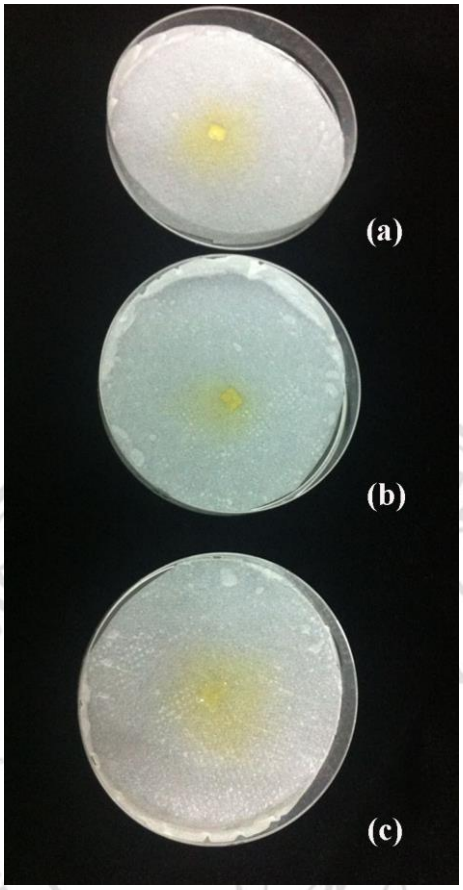

Figure 4.15 The release of propolis from electrospun fiber mats of 5% (w/v) propolis-8% (w/v) PVP K90 with (a) without Tween 80, (b) with 0.5% (w/v) Tween 80, and (c) 1% (w/v) Tween 80.

4.3.3 IR of propolis-PVP electrospun fibers

FTIR spectra of propolis-PVP electrospun fibers are shown in Figure 4.16. Characteristic peaks of PVP K90 at 2594 cm^{-1} (C-H stretching), 1644 cm^{-1} (C=O stretching), 1461 cm^{-1} (C-H beading of CH_2) and 1285 cm^{-1} (C-N stretching) are shown in Figure 4.16 (a). Due to the hydrophilic nature of PVP, a broad band was observed (O-H stretch) at about 3500 cm^{-1} [89,90]. The spectrum of propolis shows characteristic peaks at 1681 cm^{-1}, 1628 cm^{-1}, 1599 cm^{-1}, 1374 cm^{-1}, 1257 cm^{-1}, 1162 cm^{-1} and 1058 cm^{-1} in Figure 4.16 (b) which correspond to functional groups of flavonoids, lipids and alcohol groups in propolis extract [91,92]. Figure 4.16 (c), 4.16(d) and 4.16 (e) show characteristic peaks of electrospun fibers of 8% (w/v) PVP K90 with 5% (w/v) propolis without additive, with additives and with additives including Tween 80, respectively. The main peaks of PVP K90 and propolis can also be observed in these spectra. The strong absorption of the carbonyl group stretching at 1644 cm^{-1} shifted to 1651 cm^{-1} (Figure 4.16 (c), (d), (e)). The result indicated the existence of weak chemical bonding between the band of C=O of PVP K90 and propolis.

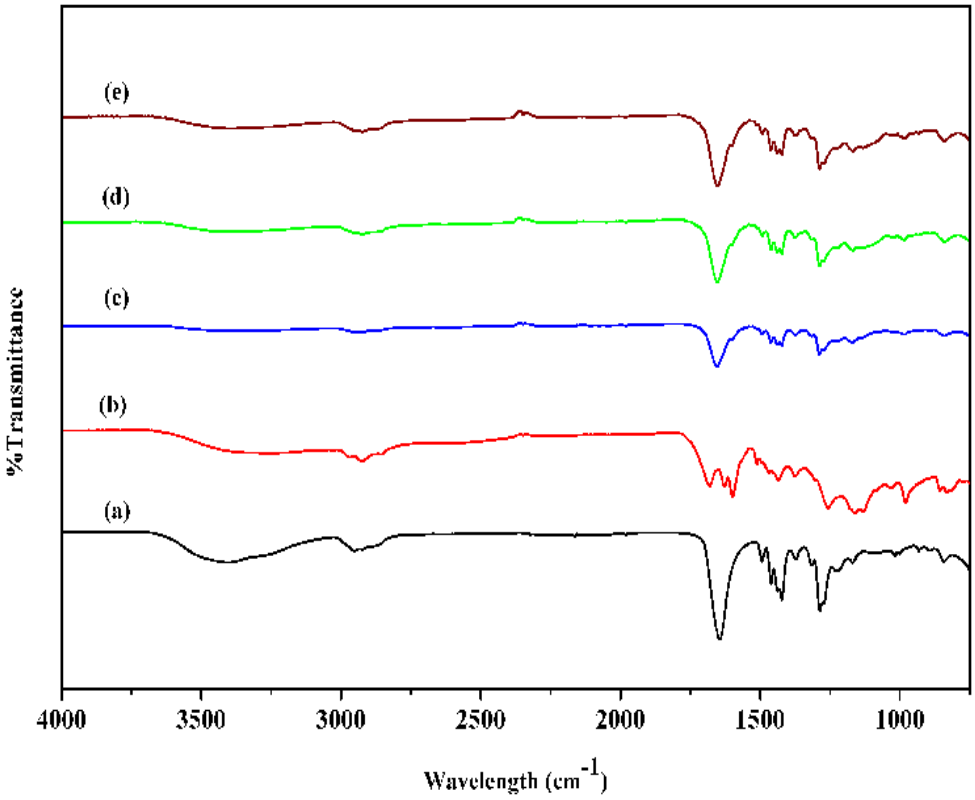

Figure 4.16 FTIR spectra: (a) PVP K90, (b) propolis extract, (c) PVP K90 electrospun fibers with 5% (w/v) propolis, (d) PVP K90 electrospun fibers with 5% (w/v) propolis with additives and (e) PVP K90 electrospun fibers with 5% (w/v) propolis with additives and 1% (w/v) Tween 80.

4.3.4 DSC of propolis-PVP electrospun fibers

DSC thermatograms of the electrospun fibers of 8% (w/v) PVP K90 with 5% (w/v) propolis without additives, with additives and with additives including Tween 80 are shown in Figure 4.17 (c), (d) and (e) and compared with raw material of PVP K90 and propolis in Figure 4.17 (a) and (b), respectively. Raw material of PVP K90 showed a broad endothermic curve. Electrospun fibers of PVP K90 with propolis had also an endothermic curve but became obtuse and the peak shifted toward the lower temperature. These results show that the crystalline microstructure of electrospun fibers did not form. This was so because the majority of the chains are in the non-crystalline state due to the rapid solidification process of stretched chains during the electrospinning process [93].

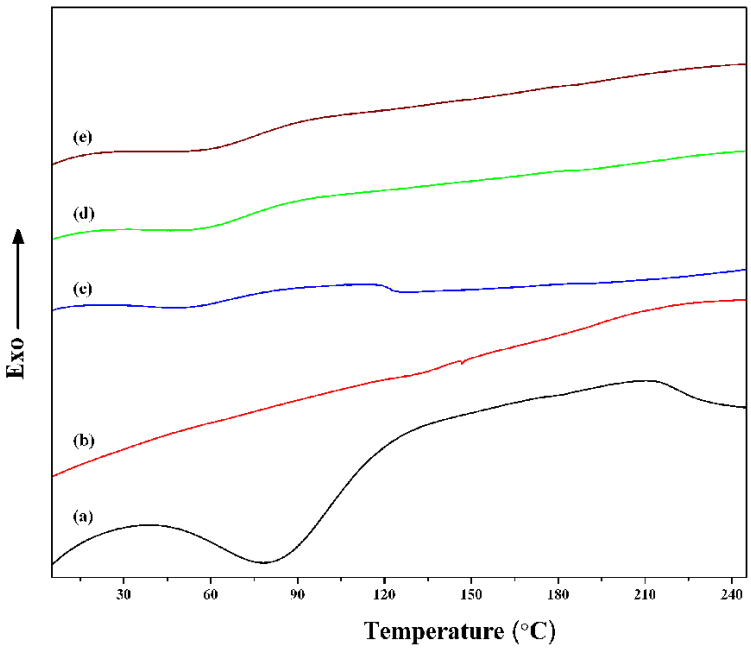

Figure 4.17 DSC thermograms: (a) PVP K90, (b) propolis extract, (c) PVP K90 electrospun fibers with 5% (w/v) propolis, (d) PVP K90 electrospun fibers with 5% (w/v) propolis with additives, and (e) PVP K90 electrospun fibers with 5% (w/v) propolis with additives and 1% (w/v) Tween 80.

4.3.5 XRD of propolis-PVP electrospun fibers

XRD patterns of propolis, PVP K90 and electrospun fibers of PVP K90 with propolis and additives are compared in Figure 4.18. There are no peaks of crystalline in the XRD patterns of the electrospun fibers of PVP K90 with propolis and additives (Figure 4.18 (c), (d) and (e)), indicating that all of the materials in the electrospun fibers were in an amorphous state and the electrospinning process did not lead to the development of the crystalline microstructures of electrospun fibers. These XRD results were supported by DSC analysis.

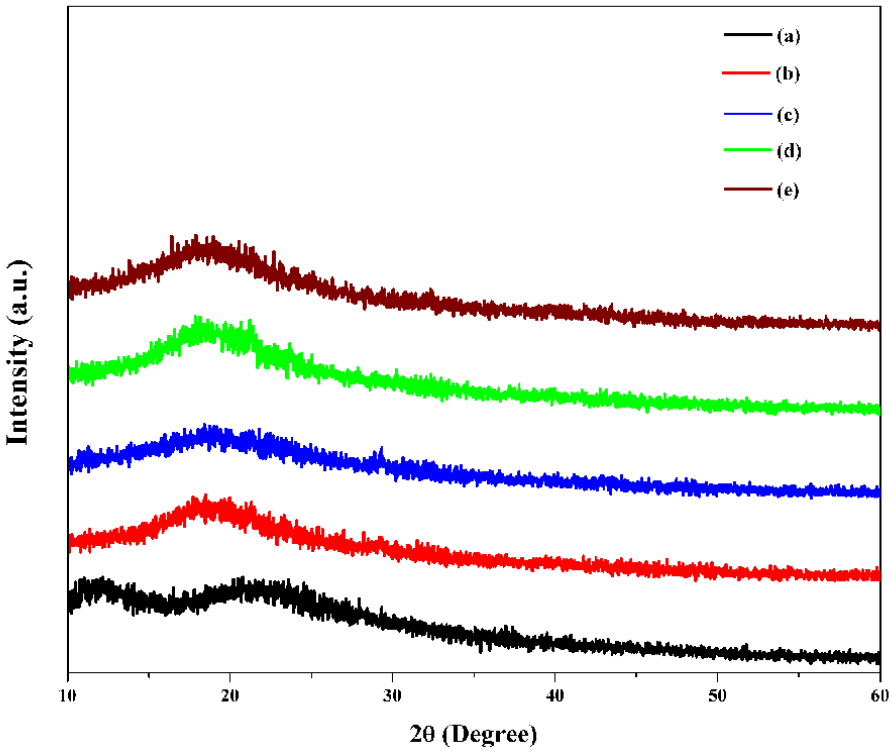

Figure 4.18 XRD patterns: (a) PVP K90, (b) propolis extract, (c) PVP K90 electrospun fibers with 5% (w/v) propolis, (d) PVP K90 electrospun fibers with 5% (w/v) propolis with additives, and (e) PVP K90 electrospun fibers with 5% (w/v) propolis with additives and 1% (w/v) Tween 80.

4.4 Determination of content of propolis in electrospun fibers

A UV-vis spectrophometer was used to determine the content of propolis incorporated in the electrospun fibers. The various amounts of propolis extract were dissolved in absolute ethanol and quantitatively analyzed by using the UV spectrophotometry method. The UV-vis spectra of the ethanol extracts are similar to typical polyphenol spectra. Normally, these spectra form a broad band around 280-330 nm [91]. A maximum wavelength at 300 nm (as seen in appendix B) was used in this study to determine the content of propolis in electrospun fibers. The calibration curve was plotted between the concentration of propolis extract and the UV absorbance, as shown in Figure 4.19. The samples of propolis-PVP electrospun fibers from different areas of the fiber mats were weighted accurately, dissolved in absolute ethanol to calculate the dry basis weight of electrospun fibers that is equivalent to the final concentration of propolis at 1mg%. The content of propolis in electrospun fibers was calculated from the linear equation of calibration curve ($y = 0.3907x + 0.0073$, $R^2 = 0.999$), (n=6).

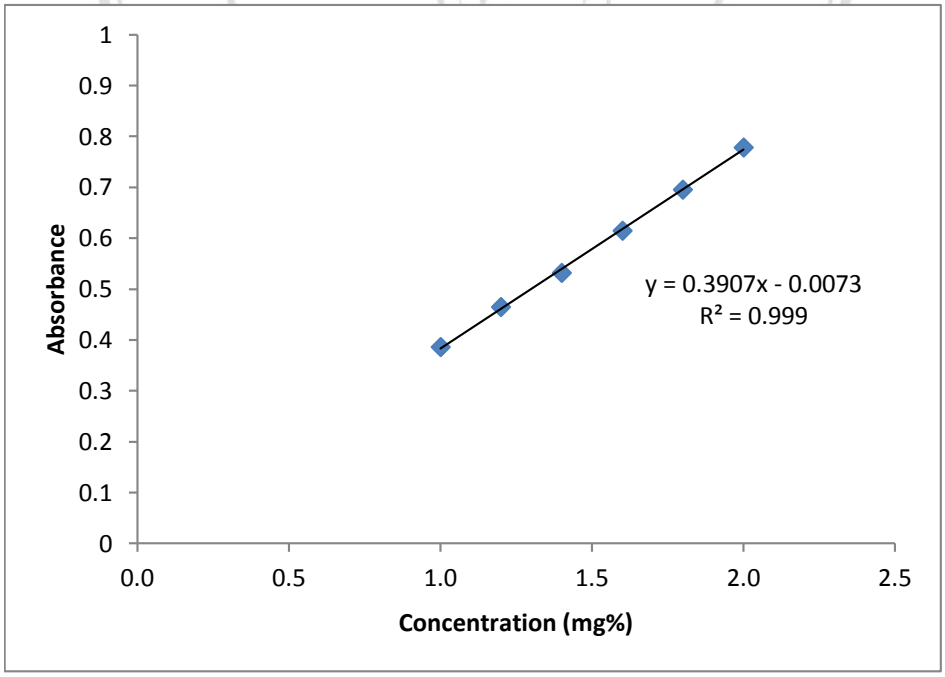

Figure 4.19 Calibration curve of propolis in absolute ethanol as analyzed by UV spectrophotometry at 300 nm.

Four formulations of propolis-PVP electrospun fibers were chosen to determine content and uniformity of propolis in the propolis-PVP electrospun fibers. The results are presented in Table 4.11. For all the formulations the content of propolis was more than 96%. The results confirmed that propolis incorporated in the polymer solution can stand the electrospinning process and has a good distribution by rendering a good uniformity of the propolis content.

Table 4.11 Content of propolis in propolis-PVP K90 electrospun fibers using different formulations.

Formulation	Content (%) of propolis	Amount of propolis (mg) in each of electrospun fiber mats (26-28 mg of tested sample)
8% (w/v) PVP K90 + 5% (w/v) propolis electrospun fibers	99.75 ± 1.21	1.00 ± 0.01
8% (w/v) PVP K90 + 5% (w/v) propolis with additives electrospun fibers	96.82 ± 0.92	0.97 ± 0.01
8% (w/v) PVP K90 + 5% (w/v) propolis + 1% (w/v) Tween 80 electrospun fibers	97.32 ± 0.93	0.97 ± 0.01
8% (w/v) PVP K90 + 5% (w/v) propolis + 1% (w/v) Tween 80 with additives electrospun fibers	98.53 ± 1.71	0.99 ± 0.02

It was observed that electrospun fibers which deposited and accumulated as mats on the collector had different thicknesses of the mats in different areas. Since the syringe used in the electrospinning process in this study was fixed, the fibers were spread from the middle of the jet path before attaching themselves to the collector. The thicknesses of the electrospun fibers mats became less from the middle of the jet path. To proof this observation, equal areas of electrospun fibers (0.5 x 0.5 cm) were cut, dissolved in absolute ethanol, diluted to the appropriate concentration and the content of propolis was analyzed using the same technique as mentioned earlier. The samples of electrospun fiber mats from the middle area were compared with those located at the area that was 5 cm away from the middle area. The results showed that the amount of propolis from the electrospun fiber mats from the middle area produced thicker mats showing a higher amount of propolis when compared with a sample of electrospun fiber mats from the 5 cm far from the middle area, as shown in Table 4.12. It was concluded that cutting into mats that calculated as weight of electrospun fibers was the preferred method to obtain the targeted amount of propolis to make a comparative study of the antimicrobial activities from propolis-PVP K90 electrospun fibers.

Table 4.12 Amount of propolis in propolis-PVP K90 electrospun fibers from different mat areas.

Formulation	Amount of propolis (in mg) in each of electrospun fiber mats	
	At the middle path	At an area 5 cm away from the middle of the mats
8% (w/v) PVP K90 + 5% (w/v) propolis electrospun fibers	1.89 ± 0.04	1.39 ± 0.18
8% (w/v) PVP K90 + 5% (w/v) propolis with additives electrospun fibers	1.72 ± 0.02	1.17 ± 0.04

4.5 Antibacterial activities of Propolis-PVP electrospun fibers

4.5.1 Minimum Inhibitory Concentration (MIC) and Minimum Bactericidal Concentration (MBC) of propolis extract on *S. mutans*

As a preliminary study for the inhibition of bacterial growth, antibacterial activity of the propolis extract against *S. mutans* was investigated by the agar disc diffusion technique. *S. mutans* was exposed to 37.5, 75, 150, 300 and 600 mg/ml of propolis extract that was completely dissolved in DMSO. The results are shown in Figure 4.20. Propolis extract showed antibacterial activity against *S. mutans* in a dose-dependent manner by increasing the inhibition zone at 12.25 ± 0.96 mm, 12.50 ± 1.29 mm, 14.25 ± 0.50 mm, 15.50 ± 1.29 mm and 15.75 ± 1.26 mm, respectively. For MIC and MBC determination, propolis extract showed an MIC at 1.172 mg/mL and an MBC at 4.688 mg/mL. Chlorhexidine, the most powerful antibacterial activity against *S. mutans,* was used as positive control in this study and showed MIC and MBC values of 0.0039 mg/mL. The MIC and MBC values of propolis extract were different depending on the technical approaches taken by the different laboratories, bacteria strains and the variation of chemical compositions of propolis extract. The chemical components in propolis extract differ significantly according to its geographical and botanical origins affecting the different antibacterial efficiencies of propolis extract from various sources [94,95]. The Propolis antibacterial property has been attributed to its phenolic compounds, especially flavonoids, phenolic acids and their esters [96]. General evidence shows the presence of different flavonoids and the inhibitory effects of propolis on cariogenic bacteria [70,97].

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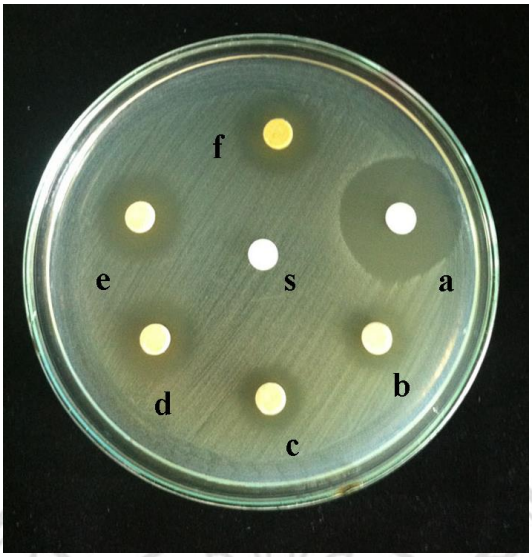


Figure 4.20. The inhibition zone of various propolis concentrations against *S. mutans*.

(Note: a = chlorhexidine solution, b = propolis extract of 37.5 mg/mL, c = propolis extract of 75 mg/mL, d = propolis extract of 150 mg/mL, e = propolis extract of 300 mg/mL, f = propolis extract of 600 mg/mL and s = DMSO)

4.5.2 Antibacterial activity of propolis-PVP electrospun fibers

In the present study, examination of antibacterial activities of fast dissolving electrospun fibers against *S. mutans* was performed by applying a paper disc method. The propolis-PVP K90 electrospun fibers were weighed in the amount equivalent to 1, 2.5, 5, 7.5, 10 and 15 MIC when dissolved in the solvent (1 MIC of propolis extract = 1.172 mg/mL).

At low concentrations of propolis extract, electrospun fibers did not show the inhibition zone on BHI nutrients after being tested with bacteria. The inhibition zones showed, however, when the concentration of propolis extract was used at 10 MIC and more than MIC. For further study, the antibacterial activity of propolis extract and electrospun fibers that incorporated propolis at 10 and 15 MIC, were compared.

Figure 4.21 and Figure 4.22 show the inhibition zone of propolis extract and electrospun fibers with propolis in the amount of 10 and 15 MIC when dissolved in DMSO and sterile distilled water, respectively. Pure solvent that is used for dissolving the samples and 0.12% chlorhexidine mouthwash (product A) were used as negative and positive control, respectively. The results of the inhibition zone are concluded in Table 4.13.

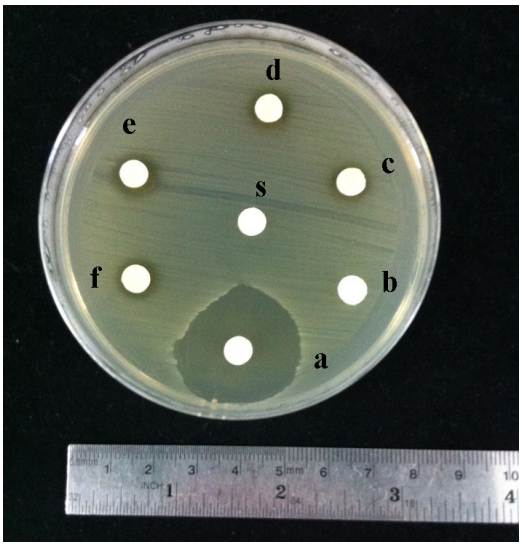


Figure 4.21 Antibacterial activity (zone of inhibition) of propolis extract and propolis-PVP K90 electrospun fibers on *S. mutans* when dissolved in DMSO

(Note: a = chlorhexidine solution (product A), b = mouthwash solution (product C), c = 8% (w/v) PVP K90 with 5% (w/v) propolis electrospun fibers at 15 MIC, d = 8% (w/v) PVP K90 with 5% (w/v) propolis electrospun fibers at 10 MIC, e = propolis extract at 10 MIC, f = propolis extract at 15 MIC, s = DMSO)

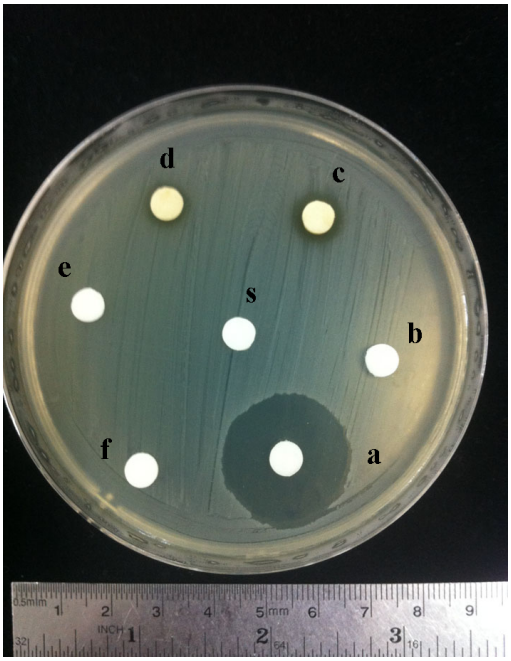


Figure 4.22 The antibacterial activities (zone of inhibition) of propolis extract and propolis-PVP K90 electrospun fibers on *S. mutans* when dissolved in water

(Note: a = chlorhexidine solution (product A), b = mouthwash solution (product C), c = 8% (w/v) PVP K90 with 5% (w/v) propolis electrospun fibers at 15 MIC, d = 8% (w/v) PVP K90 with 5% (w/v) propolis electrospun fibers at 10 MIC, e = propolis extract at 10 MIC, f = propolis extract at 15 MIC, s = sterile distilled water)

Table 4.13 The inhibition zone of the propolis extract and propolis electrospun fibers tested on antibacterial activities on *S. mutans*

Sample	Inhibition zone (mm)	
	DMSO (solvent)	Sterile distilled water (solvent)
Control	0	0
Propolis extract at 10 MIC	9.40 ± 0.36	0
Propolis extract at 15 MIC	12.17 ± 0.29	0
Propolis electrospun fibers at 10 MIC	8.53 ± 0.42	7.67 ± 0.29
Propolis electrospun fibers at 15 MIC	9.33 ± 0.29	8.40 ± 0.17
Chlorhexidine mouthwash solution (product A)	N/A	24.67 ± 0.29
Chlorhexidine electrospun fibers	N/A	23.83 ± 0.29

Besides 0.12% chlorhexidine mouthwash (product A), 5 different mouthwash products (product B, C,D,E and F) from the market were investigated on their antibacterial activity against *S. mutans* and the results are shown in Table 4.14.

Table 4.14. The inhibition zone of the 6 commercial mouthwash solutions tested on antibacterial activities on *S. mutans*

Mouthwash product	Inhibition zone (mm)
Product A (Chlorhexidine mouthwash)	24.67 ± 0.29
Product B	14.16 ± 0.29
Product C	0
Product D	0
Product E	0
Product F	0

The results of this study on antibacterial activities against *S. mutans* of propolis extract compared with propolis electrospun fibers, demonstrated that propolis extract did not dissolve in water. On the other hand, propolis extract showed good solubility in DMSO and showed an inhibition zone of 9.40 ± 0.36 and 12.17 ± 0.36 mm for propolis extract concentrations that are equivalent to 10 MIC and 15 MIC, respectively. The electrospinning process produces fibers with small diameters with a very high porosity but also promotes high solubility; as PVP is a polymer that is hygroscopic and has hydrophilic properties, a polymer chain of electrospun fibers can absorb water and dissolves rapidly [28,42]. The results as summarized in Table 4.13, show that propolis electrospun fibers that were loaded with an amount of propolis equivalent to 10 and 15 MIC could not be dissolved completely in water. This might be because the propolis component usually contains resins and waxes that are not water-dissolvable substances [98]. From the microstructure, propolis could be released from the PVP K90 polymer

base thus revealing an inhibition zone of 7.67 ± 0.29 and 8.40 ± 0.17 mm from the fibers at an amount of propolis equivalent to 10 and 15 MIC, respectively. Increasing the amount of propolis from 10 MIC to 15 MIC significantly, increased the inhibition zone against *S. mutans* ($p < 0.05$). In case DMSO is used as a solvent, both the propolis extract and the propolis electrospun fibers dissolved completely and all of them showed an inhibition zone as shown in Table 4.13. Propolis electrospun fibers revealed smaller inhibition zones when compared to the inhibition zone of propolis extracts that dissolve with the same solvent and the same concentration of propolis. When the fiber mats were dissolved, polymer PVP K90 in the fibers could be dissolved. This generated a highly vicious layer that could be trapped and retarded propolis to diffuse and expose the bacteria. The same results were obtained when comparing the inhibition zone of a 0.12% chlorhexidine solution with chlorhexidine electrospun fibers at the same amount of chlorhexidine in the fibers, as shown in Figure 4.23. The defined dissolution rate (dX/dt) could be explained by using the Noyes-Whitney equation [99,100] as follows:

$$dX/dt = A \times D / \delta \times (C_0 - X / V)$$

In the above, X is the amount of drug in the solution, t is time, A is surface area, D is diffusion coefficient of the drug, δ is effective diffusion boundary layer, C_0 is saturation solubility of the drug and V is volume of dissolution medium. Even though the structure of fiber mats have a high surface area and porosity that increases the dissolution rate , PVP K90 fibers that adsorbed water and dissolved in small amounts of water created viscous layers with an important thickness of the diffusion layer. When this layer is more viscous, it retards the dissolution and diffusion of propolis molecules to dissolution medium. The propolis electrospun fibers were wetted and disintegrated rapidly in small amounts of water as mouth dissolve fibers mats, but the hydrophobicity property of propolis and viscous layer of soluble polymer retarded the dissolution of the fibers.

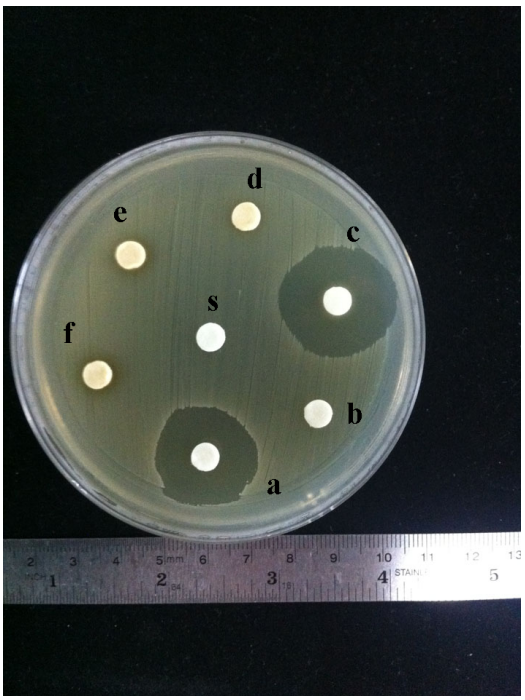


Figure 4.23 The antibacterial activity (zone of inhibition) of chlorhexidine solution, chlorhexidine electrospun fibers and propolis-PVP K90 electrospun fibers on *S. mutans* (when electrospun fibers are dissolved in sterile distilled water)

(Note: a = chlorhexidine electrospun fibers, b = mouthwash solution (product C), c = chlorhexidine solution (product A), d = 8% (w/v) PVP K90 with 5% (w/v) propolis electrospun fibers at 5 MIC, e = 8% (w/v) PVP K90 with 5% (w/v) propolis electrospun fibers at 10 MIC, f = 8% (w/v) PVP K90 with 5% (w/v) propolis electrospun fibers at 15 MIC, s = sterile distilled water)

Antimicrobial activities of 6 commercial mouthwash solutions are shown in Table 4.14. A 0.12% chlorhexidine mouthwash solution (product A) showed the highest antibacterial activity against *S. mutans*. Product B, which contains o-Cymen-5-ol and dipotassium glycerrhizate as active ingredients, showed a smaller inhibition zone when compared with product A. Other products of mouthwash solutions (product C-E) that contained volatile oils with or without sodium fluoride or natural extracts, did not show the inhibition zone. There are various reports showing that chlorhexidine is the most effective antimicrobial agent used for oral cavities[101,102]. Volatile oils, such as eucalyptol, methyl salicylate, thymol, menthol are antiseptic agents have low antibacterial activity when compared with chemical substances. No inhibition zone was found in the mouthwash products that contained volatile oils or natural extract, which was due to the low concentration of these substances in the formulation that was below the MIC that can withstand be against *S. mutans* [103]. In this study, additives such as menthol, thymol, eucalyptus oil and methyl salicylate were incorporated in the electrospun fibers as flavoring agents.

It was found that electrospun fibers with propolis that had poor wettability cannot dissolve completely in water. For this reason, 1% *(w/v)* of Tween 80 was used as wetting agent by adding it to the propolis-PVP K90 solution to improve wettability of the electrospun fibers. The results of antibacterial activity from these formulations are shown in Figure 4.24 and a summary of the inhibition zone for testing antibacterial activity from propolis-PVP K90 electrospun fibers is shown in Table 4.15

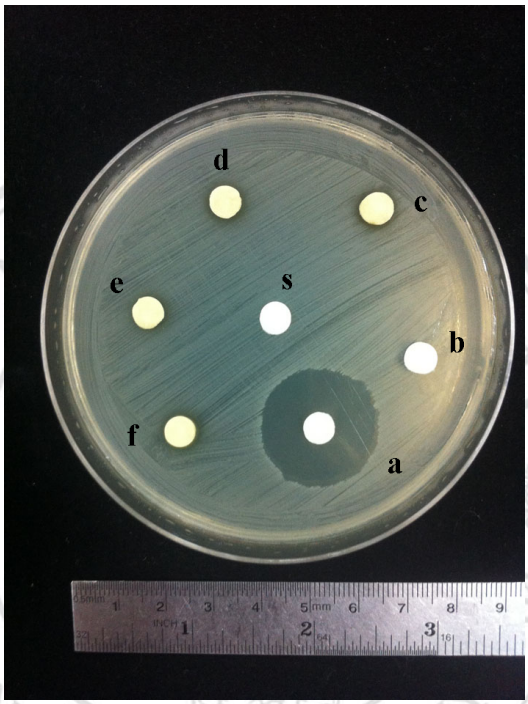


Figure 4.24 The antibacterial activity (zone of inhibition) of propolis-PVP K90 electrospun fibers with additives with/without Tween 80 as wetting agent on *S. mutans* (when dissolved in sterile distilled water)

(Note: a = chlorhexidine solution (product A), b = mouthwash solution (product C), c = 8% (w/v) PVP K90 with 5% (w/v) propolis with additives electrospun fibers at 15 MIC, d = 8% (w/v) PVP K90 with 5% (w/v) propolis with additives electrospun fibers at 10 MIC, e = 8% (w/v) PVP K90 with 5% (w/v) propolis with additives and 1% (w/v) Tween 80 electrospun fibers at 10 MIC, f = 8% (w/v) PVP K90 with 5% (w/v) propolis with additives and 1% (w/v) Tween 80 electrospun fibers at 15 MIC, s = sterile distilled water)

Table 4.15 The inhibition zone of the propolis-PVP electrospun fibers included additives with/without Tween 80 tested for antibacterial activity on *S. mutans*.

Formulations	Inhibition zone (mm)
Propolis electrospun fibers at 10 MIC	7.67 ± 0.29
Propolis electrospun fibers at 10 MIC with additives	7.67 ± 0.29
Propolis electrospun fibers at 10 MIC with additives and Tween 80	7.00 ± 0.00
Propolis electrospun fibers at 15 MIC	8.40 ± 0.17
Propolis electrospun fibers at 15 MIC with additives	8.27 ± 0.25
Propolis electrospun fibers at 15 MIC with additives and Tween 80	7.00 ± 0.00*

* Indicates a significant difference ($p < 0.05$) between the formulations that contained the same amount of propolis.

A small amount of volatile oils as flavoring agent did not affect the antibacterial activity against *S. mutans* when compared with propolis electrospun fibers without the additives. There was no statistical difference when the inhibition zones of propolis electrospun with additives and without additives were compared. The results showed that the inhibition zones for these formulations were very similar, that means no synergistic antibacterial activity from the additives. In case 1% (w/v) Tween 80 addition was used, the inhibition zone seemed to be smaller. It was found that significant decreases in the inhibition zones were found in the formation of propolis electrospun fibers at 15 MIC with additives which added 1% (w/v) Tween 80 ($p < 0.05$). Many reports have mentioned that many nonionic surfactants do not inhibit the growth and glycan synthesis of *S. mutans* [104]. A study by Tomita et al. showed that nonionic surfactants could stimulate the production of glucosyltransferase in some strains of *S. mutans* when the concentration of surfactant reached the proper concentration [105]. The incorporation of Tween 80 in the growth media also

enhanced the growth of streptococci and lactobacilli by addition of an exogenous source of fatty acid. Otherwise, Tween 80 have been associated with enhanced the acid tolerance of *S. mutans* [106-108]. For this reason, it is suggested that Tween 80, which is a nonionic surfactant used in the formulations, be used to reduce the antibacterial activity against *S. mutans*.

4.6 Inhibition of adherence of *S. mutans* to a glass surface by propolis-PVP K90 electrospun fibers

Figure 4.25 shows the effect of propolis extract and propolis electrospun fibers that are loaded with propolis at sub-MIC 0.015, 0.03, 0.04, 0.06, 0.15, 0.30, 0.60 and 1MIC on the adhesion of *S. mutans* to the glass surface. It was found that propolis extract dissolved in DMSO showed a stronger inhibitory effect than both electrospun propolis fibers dissolved in DMSO and water. The inhibitory activity increased when the concentration of propolis extract was increased. The results showed that the sub-MIC concentration of propolis that did not show antibacterial activity, could inhibit the adherence of *S. mutans* to a smooth glass surface. The results confirmed other research work that concluded that the anti- plaque properties of propolis extracts are related to glucosyltransferases activity inhibition, rather than antimicrobial action [71,109].

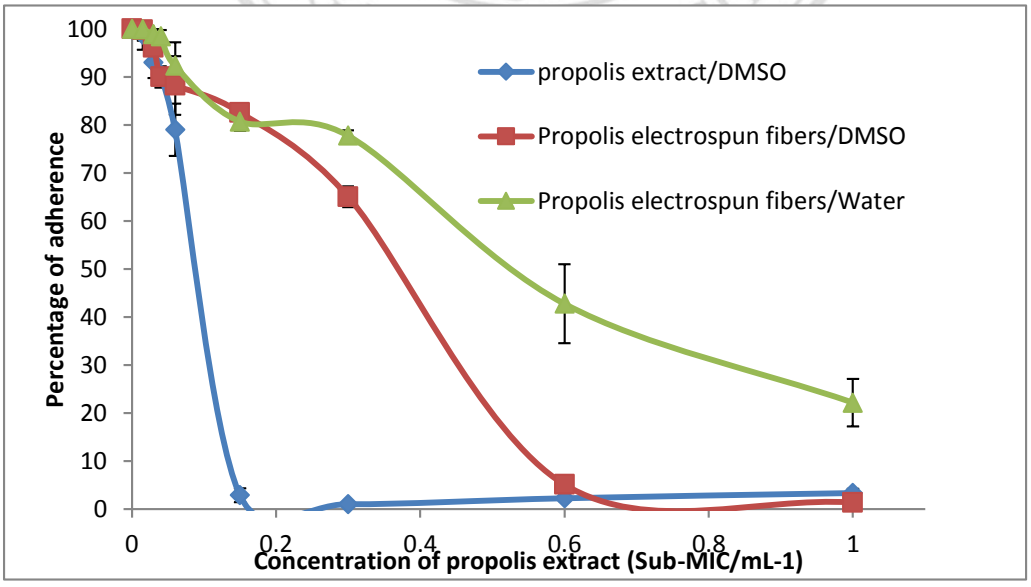


Figure 4.25 Effect of propolis extract and propolis electrospun fibers at sub-MIC on the adhesion of *S. mutans* to the glass surface.

Since mouth dissolving fibers have a limited capacity to dissolve in small amounts of liquid in oral cavities, propolis electrospun fibers dissolving in sterile distilled water were selected. Concentrations of propolis in electrospun fibers at 0.6 MIC and 1 MIC were chosen to study the efficiency of inhibition of adherence of *S. mutans* from various formulations of propolis-PVP K90 electrospun fibers. Propolis-PVP K90 electrospun fibers with/without additives and with/without Tween 80 were studied and the results are shown in Figure 4.26.

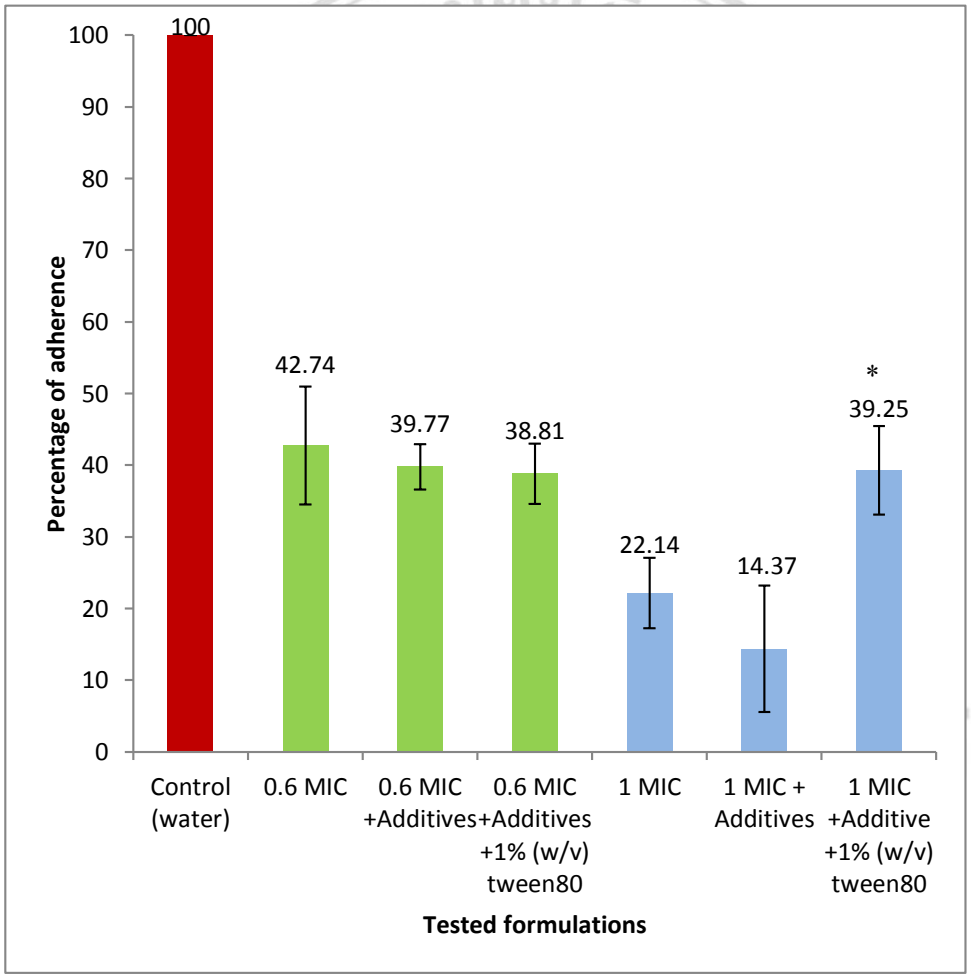


Figure 4.26 Effect of propolis electrospun fibers of various formulations on the adhesion of *S. mutans* to a smooth glass surface.

* Indicates a significant difference ($p < 0.05$) between the formulations that contained the same amount of propolis.

The results in Figure 4.26 show that the amount of propolis in propolis-PVP K90 electrospun fibers at 0.6 MIC and 1 MIC revealed a percentage of *S. mutans* adherences of about 22 - 43 % . Incorporated volatile oils as flavoring agent also reduce the amount of attached *S. mutans* on the smooth surface. Tween 80 added to the formulation of electrospun fibers to improve the wettability of the electrospun fibers seems to have no effect on the adherence of *S. mutans* as shown in the test of formulations at 0.6 MIC. There was no statistical difference of the adherence of *S. mutans* in the formulations that incorporated additives and additives with Tween 80 when compared with PVP K90 electrospun fibers that incorporated propolis extract only. However, the formulation of propolis-PVP K90 electrospun fibers at 1 MIC increased the *S. mutans* adherence on a smooth surface to 39.25%. There was a significant increase of the adherence of *S. mutans* when compared with the formulations that were free from Tween 80 ($p < 0.05$). This result conforms to previous studies and suggests that surfactant might vary the cariogenic potential of *S. mutans* even at a low concentration. Water insoluble glucan synthesis and the artificial plaque formation were significantly enhanced [105]. Following counting the CFU on agar plates from the study to determine the amount of surviving bacteria cells that adhered to the glass surface after the incubation time (T_{18}), it was confirmed that propolis-PVP K90 electrospun fibers with or without additives and Tween 80 reduced the amount of *S. mutans* as shown in Figure 4.27 . The amount of *S. mutans* from all of the tested formulations was around $4.7 \times 10^4 - 7.1 \times 10^5$ CFU/mL, whereas the amount of *S. mutans* which were treated with sterile water as blank increased to 2×10^{12} CFU/mL from the initial amount of *S. mutans* (at T_0) in all of the test was around 10^6 CFU/mL.

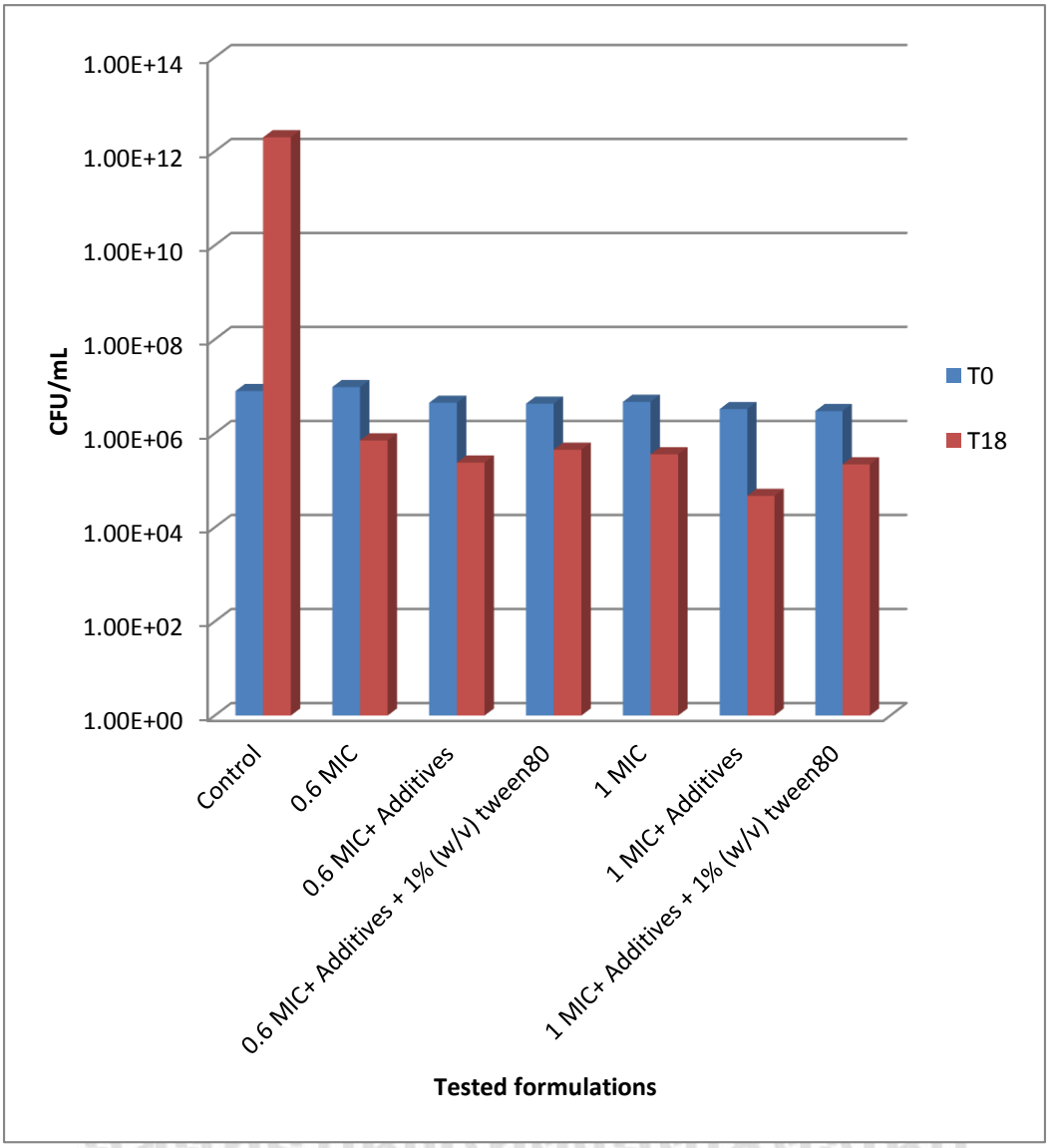


Figure 4.27 The amount of surviving *S. mutans* in plaque that adhered to the smooth glass surface using various formulations of propolis electrospun fibers after testing the adhesion of *S. mutans* on a smooth glass surface for 18 hours.

Five kinds of different commercial mouthwash products that claimed, among others, anti-plaque formation, anti-tooth decay, would reduce the accumulation of bacteria in oral cavities including chlorhexidine mouthwash solution, were tested on the inhibition of adherence of *S. mutans*. The results of the efficiency of mouthwash products on inhibition of *S. mutans* to adhere to a smooth surface are given in Figure 4.28.

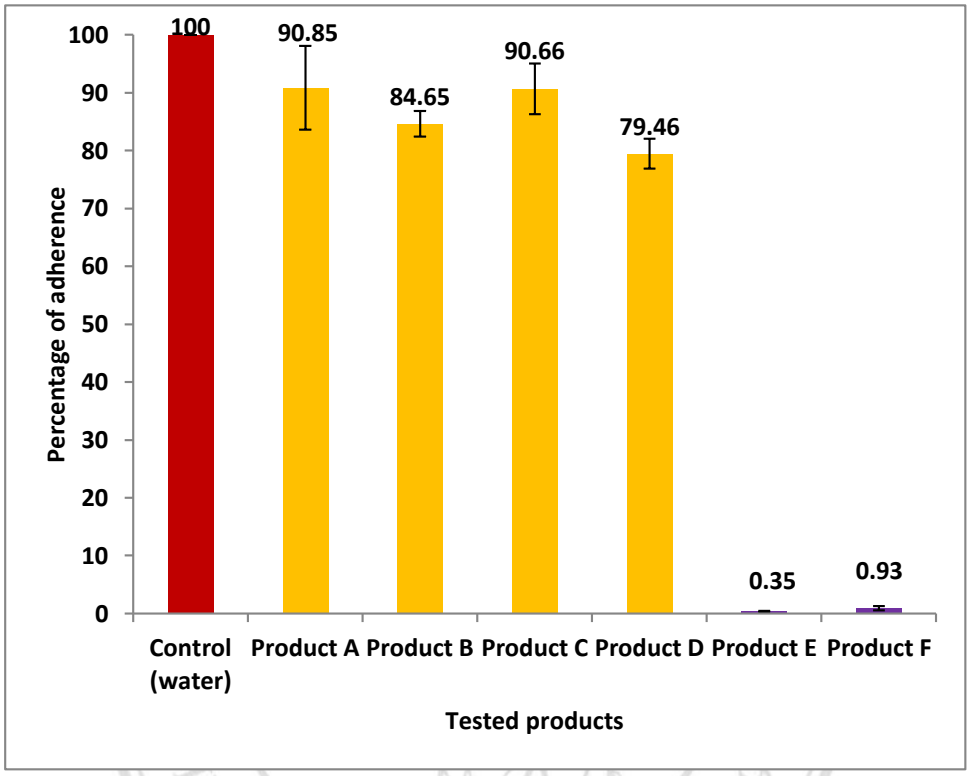


Figure 4.28 Effects of 6 commercial mouthwash solution products on cell adhesion of *S. mutans* to a smooth glass surface.

The results in Figure 4.28 showed that the mouthwash products which contained volatile oils as active ingredients (Products A, B, C and D) had less power to inhibit *S. mutans* to adhere to a smooth surface; whereas, product E which contained a chemical substance (such as o-Cymen-5-ol and dipotassium glycerrhizate) in its formulation; product F with chlorhexidine in the form of a mouthwash solution, showed very powerful action to inhibit *S. mutans* to adhere to a smooth surface. After counting the CFU on agar plates to determine the amount of survival bacteria cells that adhered to the glass surface after incubation time (T_{18}), it was confirmed that various formulations of mouthwash solutions from the market had different abilities to inhibit the growth of *S. mutans*, as shown in Figure 4.29. From the initial amount of *S. mutans* (at T_0) in all of the tests, around 10^6 CFU/mL, it was found that the amount of *S. mutans* from product A, B, C and D increased to 1 x 10^7 - 2 x 10^7 CFU/mL after 18 hours of

incubation time. The reduction of *S. mutans* with product E and F was nearly 100%, which was the effect of antibacterial activities of the chemical substances in these mouthwash solutions.

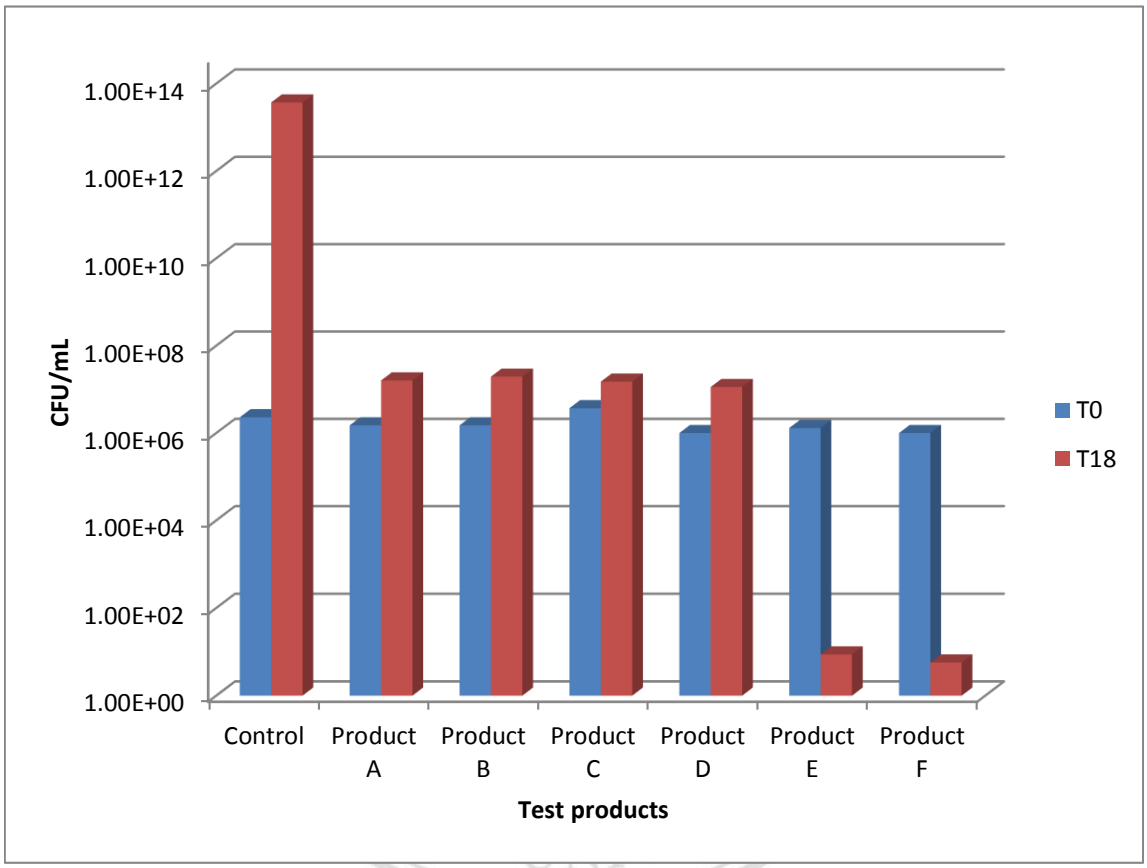


Figure 4.29. The amount of surviving *S. mutans* in plaque that adhered to the smooth glass surface from various commercial mouthwash solutions hours after testing the adhesion of *S. mutans* to a smooth glass surface for 18 hours.

To compare the efficiency of propolis-PVP electrospun fibers with other commercial mouthwash products with regard to anti-plaque formation agent, the results showed that propolis extract in electrospun fibers at 0.6 and 1 MIC were more effective when compared with the mouthwash solutions that are free from chemical antiseptic substances. The results suggest that propolis did not only reduce the adhesion of *S. mutans* as plaque formation on a smooth surface but also reduced the number of survival *S. mutans* cells in the plaque.

4.7 Morphology study in adherence cells of *S. mutans*

4.7.1 Morphology of adherence cells of *S. matans* to the glass surface as characterized by light microscopy

The accumulation of *S. mutans* on the surface of a glass slide before crystal violet staining as control, is shown in Figure 4.30. After crystal violet staining as in Figure 4.31 (a) numerous of *S. mutans* as plaque appeared on the glass slide.

Morphological changes of bacteria cells treated with propolis-PVP K90 electrospun fibers in Figure 4.31 (b) and Figure 4.31 (c) were revealed in smaller or denser biofilm, which indicated that bacteria cells were destroyed or changed and dead cells proving evidence were found through optical micrographs. Many dead bacteria were observed in the optical image of the glass slide as a result of treatment with propolis.

Various studies have shown several mechanisms of propolis such as the disorganization of the cytoplasmatic membrane and the cell wall; partial bacteriolysis, formation of pseudomulticellular colonies and inhibition of protein synthesis that influences gaining antibacterial activities and anti-plaque formation [110,111].

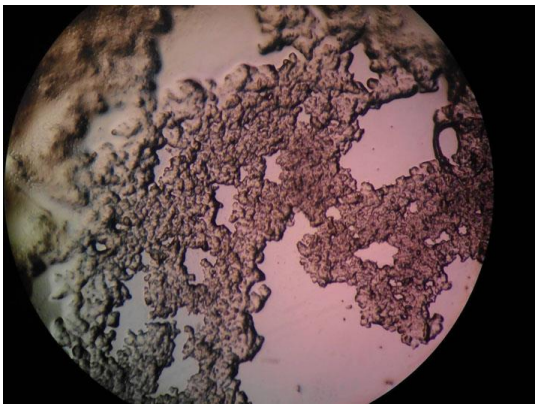


Figure 4.30 Light microscopy of plaque formation of *S. mutans* before crystal violet staining.

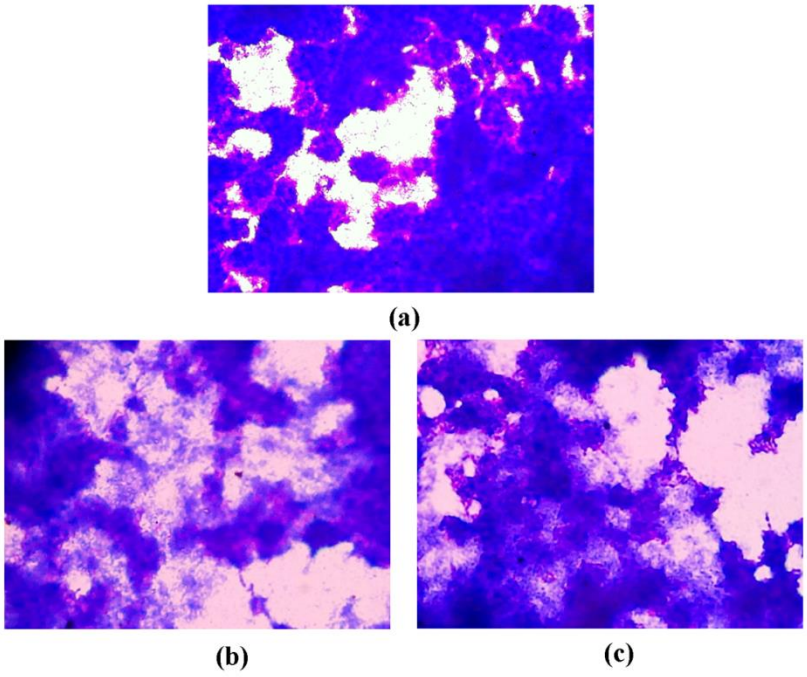


Figure 4.31 Photographs from optical microscopy of the adhesion of *S. mutans* cells to smooth glass surface after crystal violet staining compared with (a) Blank, Sterile distilled water, (b) 0.6 MIC of propolis with additives and Tween 80 electrospun fibers and (c) 0.6 MIC of propolis from propolis electrospun fibers.

4.7.2 Morphology of adherence cells of *S. mutans* to the glass surface a characterized by a SEM

The results as shown in Figure 4.32 support that propolis electrospun fibers have an antibacterial activity against *S. mutans* and an inhibitory effect on the formation of *S. mutans* biofilm.

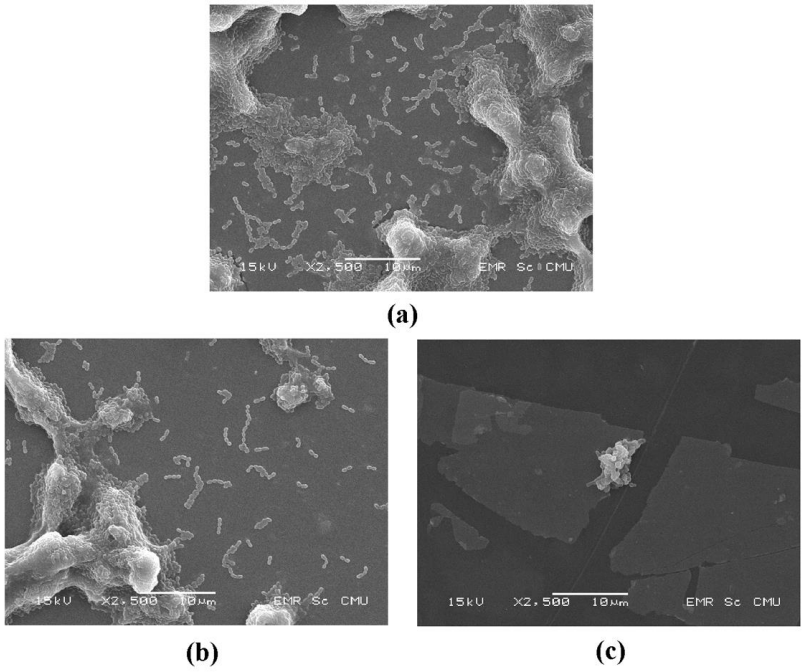
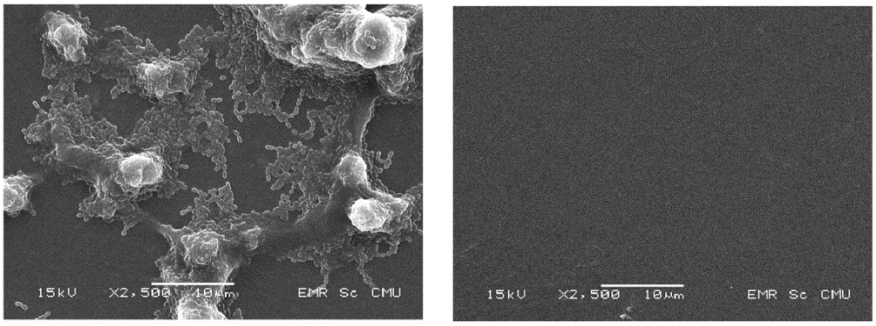


Figure 4.32 SEM photographs of the adhesion of *S. mutans* cells on smooth glass surface with (a) blank, sterile distilled water, (b) 0.6 MIC of propolis from propolis with additives and Tween 80 electrospun fibers and (c) 0.6 MIC of propolis from propolis electrospun fibers.

The photographs showing morphological changes of bacteria cells support the mechanism of propolis with regard to antibacterial activity and the cause of damage in bacteria cells. The amount of bacteria cells that were attached on glass surface was reduced in the samples of propolis electrospun fibers.



(a) **(b)**

Figure 4.33 SEM photographs of the adhesion of *S. mutans* cells to a smooth glass surface from (a) a mouthwash solution (product C) and (b) a chlorhexidine solution.

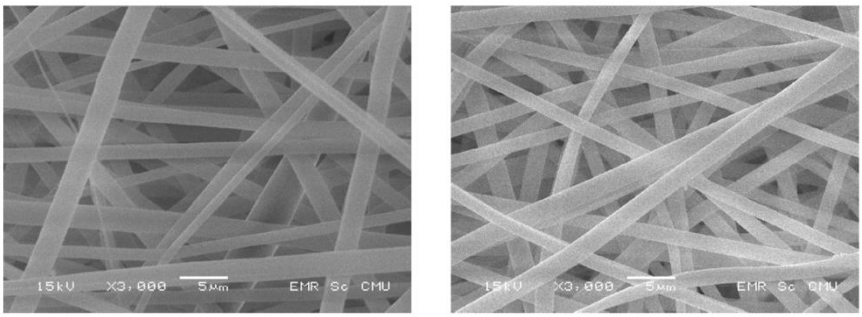
When comparing the efficiency of the mouthwash solution products, Figure 4.33 (a) shows that many bacteria cells attached to the smooth glass surface indicating less efficiency to prevent the adherence of *S. mutans* to a smooth surface as plaque formation, whereas a powerful antibacterial agent as chlorhexidine solution shows no bacterial cell adherence to a smooth surface because *S. mutans* were eradicated by this solution.

4.8 Stability test

Propolis-PVP electrospun fibers mats were stored in aluminum pouches and kept in a dry place, at room temperature, for 3 months. At the end of the storage, the samples were tested on their stability.

4.8.1 Morphology of propolis-PVP K90 electrospun fibers

SEM photographs show that electrospun fibers from all of the formulations had good morphology of electrospun fibers (Figure 4.34). The results showed that aluminum foil was an appropriate packaging to protect the electrospun fibers from moisture.



(a) (b)

Figure 4.34 SEM images of propolis-PVP K90 electrospun fibers kept for 3 months (a) 8% (w/v) PVP K90 with 5% (w/v) propolis and (b) 8% (w/v) PVP K90, 5% (w/v) propolis with additives and 1% (w/v) Tween 80.

4.8.2 Physical properties of propolis-PVP K90 electrospun fibers

XRD patterns show that all the samples of propolis-PVP K90 electrospun fibers had an amorphous form. This result revealed that all of the components in electrospun fibers were still in amorphous form. That means no ingredient changed to a crystalline form during the period of stability testing. Results from IR and DSC were similar to the samples taken at the beginning.

4.8.3 Content of propolis in propolis-PVP electrospun fibers

Two formulations of propolis-PVP electrospun fibers in the form of the finished products for oral fast dissolving electrospun fibers ((a): 8% (w/v) PVP K90 + 5% (w/v) propolis with additives electrospun fibers and (b): 8% (w/v) PVP K90 + 5% (w/v) propolis + 1% (w/v) Tween 80 with additives electrospun fibers) were chosen for content analysis. The content of propolis from propolis-PVP K90 electrospun fibers that were stored for 3 months had slightly decreased and were not significantly different when compared with the content of propolis that was kept on day 0 for stability testing ($p < 0.05$) as shown in Figure 4.35.

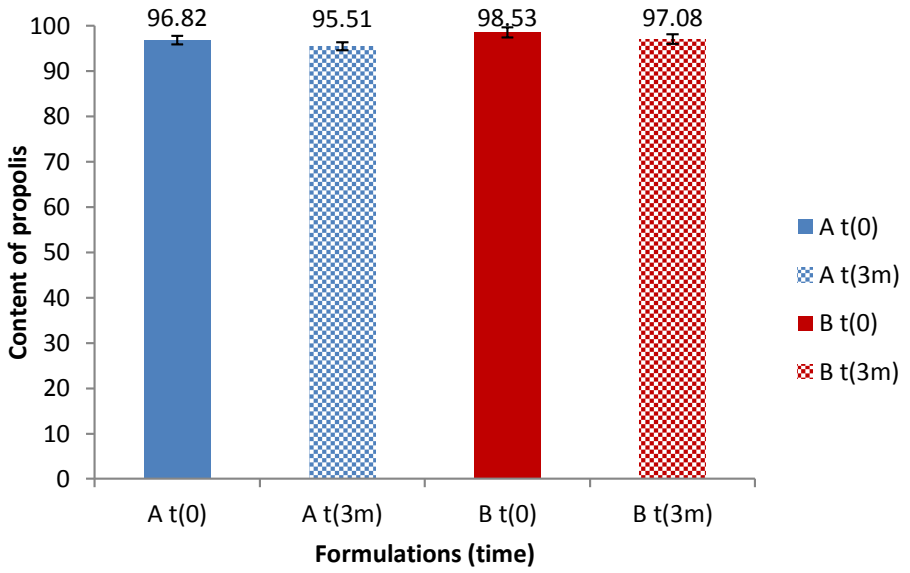


Figure 4.35 Percent content of propolis in propolis-PVP electrospun fibers after storage of 3 months; A t(0): 8% (w/v) PVP K90 + 5% (w/v) propolis with additives electrospun fibers before storage, A t(3m): 8% (w/v) PVP K90 + 5% (w/v) propolis with additives electrospun fibers after storage of 3 months, B t(0): 8% (w/v) PVP K90 + 5% (w/v) propolis + 1% (w/v) Tween 80 with additives electrospun fibers before storage and B t(3m): 8% (w/v) PVP K90 + 5% (w/v) propolis + 1% (w/v) Tween 80 with additives electrospun fibers after storage for 3 months.

4.8.4 Antibacterial activity of propolis-PVP electrospun fibers

Both 2 formulations of propolis-PVP electrospun fibers as the finished products for oral fast dissolving electrospun fibers (8% (w/v) PVP K90 + 5% (w/v) propolis with additives electrospun fibers and 8% (w/v) PVP K90 + 5% (w/v) propolis + 1% (w/v) Tween 80 with additives electrospun fibers) that were chosen for testing, showed an inhibition zone at 7.43 ± 0.40 mm and 7.07 ± 0.12 mm, respectively. These inhibition zones were similar to the inhibition zones of the freshly prepared propolis-PVP electrospun fibers.

4.8.5 Inhibition of adherence of *S. mutans* by propolis-PVP K90 electrospun fibers

Both 2 formulations of propolis-PVP electrospun fibers as finished products for oral fast dissolving electrospun fibers (8% (w/v) PVP K90 + 5% (w/v) propolis with additives electrospun fibers and 8% (w/v) PVP K90 + 5% (w/v) propolis + 1% (w/v) Tween 80 with additives electrospun fibers) chosen for testing, showed adherence percentages of *S. mutans* to a smooth surface at 42.38 ± 8.14 % and 43.30 ± 2.28 %, respectively. The adherence percentage seems to have slightly increased when compared with freshly prepared electrospun fibers with the same formulations but were not significantly different in terms of inhibition of adherence of *S. mutans* to a smooth glass surface ($p < 0.05$).

Taking all stability testing together, it may be concluded that propolis-PVP K90 electrospun fibers has a good stability during the period of testing. The propolis-PVP K90 electrospun fibers are to be kept in moisture resistance packaging such as aluminum foil pouches for unit-dose packaging to protect these from moisture.