



The Preparation of Electrospun Fiber Mats Containing Propolis Extract/CL-CMS for Wound Dressing and Cytotoxicity, Antimicrobial, Anti-Herpes Simplex Virus

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Wound dressing has developed continuously over a period of time, start by simply acting to cover the wound. The modern bandage materials are containing various active compounds for beneficial to the healing of wounds and have many techniques for preparation. The electrospinning is one of the most important techniques for wound dressing preparation. In this research, we prepared the fiber mats containing propolis extract and cross-linked carboxymethyl starch (CL-CMS) by electrospinning. All samples were prepared in three layers, the top and the base layers are poly(vinyl alcohol) (PVA) fiber mat, the middle layer is poly(vinyl pyrrolidone) (PVP)/propolis or PVP/propolis/CL-CMS. The samples were separate two groups, one group containing various amount of propolis. The propolis was varied from 0% w/v, 1% w/v, 3% w/v and 5% w/v. And another group, the samples contain fixed amount of propolis at 5% w/v and CL-CMS was varied from 0–15% w/v. The morphology of samples were observed by SEM. It was found that, they were smooth and in the medium layer, the CL-CMS powders appeared. *S. aureus*, *S. epidermidis*, *B. cereus*, MRSA and *P. aeruginosa* were antibacterial tested by using disdiffusion method, at 5% w/v of the propolis was the maximum of the antibacterial activity. Cytotoxicity and antiviral activity were tested. The CD_{50} of the sample at 0% w/v of the propolis is $794.38 \pm 0.00 \mu\text{g/ml}$, when increased the propolis concentration the CD_{50} was decrease.

Keywords: Fiber Mats, Electrospinning, Electrospun Fibers, Propolis, Antibacterial, Cytotoxicity.

1. INTRODUCTION

Nowadays the nanotechnology and nano-material is wildly studied for apply to many applications, such as electronic devices, sensors and biomedical materials.^{1,2} There are relationship between the processing, structure, properties and application as carbon nano-rods, carbon nanotube,^{3–5} and polymer nano encapsulate.⁶ In addition, the nanofiber is one of the important nanomaterials for many applications. Electrospinning is one of the most important techniques to manufacture fibers.⁷ The electrospun fibers have become popular for use as wound dressings because it is a very large surface to volume ratio that is effective for releasing active ingredients in the fibers into the skin.^{8,9} Electrospinning produces are the nonwoven

fabrics consisting of ultrafine fibers, it is highly porous. Good air flow properties will be obtained from these micro-pores and the electrospun fiber mats can protect the wound from the moisture elimination process.¹⁰ For the biomaterials in wound dressing compost of natural polymers and synthetic polymers, such as chitosan, collagen,¹¹ poly(caprolactone)¹² and poly(vinyl alcohol).⁸ Poly(vinyl alcohol) (PVA) is the synthetic polymers. It is widely used in the range of industrial, commercial, biomedical and food applications, such as lacquers, surgical threads and food-contact applications.¹³ PVA is highly biocompatible and non-toxic.¹⁴ There are kinds of antibacterial electrospun fiber mats that used for wound dressing. First, polymer that used in electrospinning technique with intrinsic antibacterial activity example is quaternized chitosan. Second, incorporation of the active ingredients into the

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polymer, such as antimicrobial agent or medical substance with polymer. Such as the electrospun fiber of poly(vinyl pyrrolidone)-iodine complex.⁹

In this study, we prepared the PVA-PVP added propolis and PVA-PVP added propolis with CL-CMS fiber mats layers. These electrospun fibers would have antibacterial activities from propolis and also more effectiveness for healing wounds because of the providing moisture to the skin. A good wound dressing in addition to protect the wound from the moisture elimination process, and then it must be able to absorb wound exudate.¹⁵ The CL-CMS is good swelling power, therefore, the CL-CMS adding to the fiber mats for the absorbed the wound exudate. The morphology, structure, and antimicrobial properties were investigated.

2. The CROSS-LINKED CARBOXY METHYL STARCH (CL-CMS) PREPARATION

The preparation of cross-linked carboxymethyl starch (CL-CMS) following the method described by Kittipongpatana.¹⁶ 40 g of monochloroacetic acid dissolved in 254 g of methanol in 3-neck round bottom flask then added the 138 g of tapioca starch followed by 75.5 g of 50% of NaOH solution. After that the 1 wt.% of epichlorohydrin was added for the cross-linking agent. The mixture was heated and controlled the temperature at 70 °C for 1 hr. Finally, the end of reaction was terminated by neutralization with glacial acetic acid. The powder was filtrated and washed several time with 80% methanol and the methanol after filtrated was tested by silver nitrate, no precipitate was found. Finally the powder was washed by 100% methanol.

3. EXTRACT OF PROPOLIS PREPARATION

The propolis was extracted by ethanolic extract. The propolis was ground and homogenized using liquid nitrogen. The 30 grams of propolis powder was dissolved in 300 ml of ethanol (70 vol.%). For ultrasonic extraction, the samples were processed in an ultrasonic chamber for 30 min, the temperature was controlled below 40 °C by ice. Finally, the solution was filtrated through a Whatman No. 1 filter paper. After that the propolis extract was evaporated the ethanol by the evaporator. Finally the propolis extract was lyophilized for removed water.

4. ELECTROSPUN FIBER MATS CONTAINING PROPOLIS EXTRACT/CL-CMS PREPARATION

The PVA-PVP/propolis nanofiber layers prepared by the 8% w/v PVA dissolved in deionized water, stirring at 80 °C for 30 min. Left to cool at room temperature and spin to the fibers by electrospinning technique for first layer. The spinning condition was as follow; 10 ml of the PVA solution was fed into a 25 ml-hypodermic syringe. The

diameter of the needle tip is 0.6 mm was used. Aluminum foil was used as the collector. The distance between the needle tips to the collector was 25 cm. The electric field of 25 kV was applied throughout the spinning process and the flow rate from 0.8 ml/hr. was employed. The spinning process was carried out at room temperature. The second layer prepared by spinning the PVP added propolis and PVP added propolis and CL-CMS particles dissolved in ethanol, stirring at room-temperature for 30 min. The PVP concentration was 8% w/v, condition one added propolis various from 0% w/v, 1% w/v, 3% w/v and 5% w/v. Condition two fixed the propolis at 5% w/v and varied the CL-CMS as 0% w/v, 5% w/v, 10% w/v and 15% w/v.

5. WATER ADSORPTION OF CL-CMS

The CL-CMS is the swelling agents, it is widely used as disintegrating agent in the tablet.¹⁷ Mechanism of swelling in the CL-CMS is caused by the absorption of water into the inner itself. From the mechanism of swelling of CL-CMS, it can be used to absorb exudate from the wound. The water adsorption of CL-CMS was defined by water swelling ratio. The CL-CMS were left at 30 °C in hot air oven for 2 days. The water swelling ratio of CL-CMS was calculated using the equation:

$$\text{water swelling ratio} = \frac{W_1 - W_0}{W_0} \times 100\% \quad (1)$$

W_0 is the dried weight of CL-CMS and W_1 is the weight of fully swollen CL-CMS. W_1 was measured after centrifuged the CL-CMS in water at 4000 rev/min for 10 min, and then the water under the CL-CMS was discarded. From the Eq. (1), the water swelling ratio of the CL-CMS is $235.67 \pm 0.24\%$

6. THE MORPHOLOGY OBSERVATION

The morphology of the electrospun fiber mats was examined using a scanning electron microscope (SEM; SNE-3000M, Nanoeye). The samples were arranged on a metal stub using carbon adhesive tape and coated with gold under vacuum before observation.

The fiber mats layers prepared by electrospinning technique. The samples were successfully prepared in three layers as top and base layer are PVA fiber mats, the middle layer is PVP added propolis or PVP added propolis and CL-CMS. The morphology of fiber mats layers were shown in Figure 1. From Figure 1(a), the morphology of PVA fibers shows that the smooth fiber with no beads. The range of fibers diameter was 200–350 nm. Figure 1(b) shows the morphology of PVP added propolis and CL-CMS. It was found that the CL-CMS powders adhere at PVP fibers.

Our research to prepare the wound dressing that can help wounds heal faster and is also active against

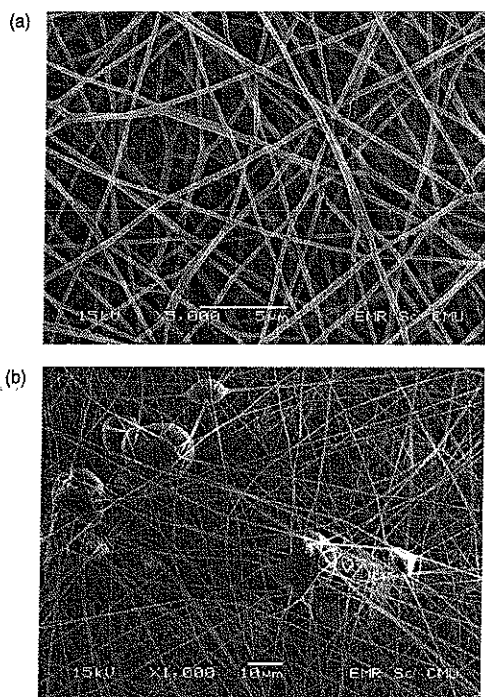


Fig. 1. The SEM image of PVA-PVP/propolis/CL-CMS powder fiber mats layers, (a) PVA fiber mats and (b) the middle layer PVP/propolis/CL-CMS powder fiber mats.

pathogenic bacteria by propolis extracts. From our unpublished research, we prepared the PVA added propolis extracted, it was found that the PVA and propolis cannot dissolve in the same solvent. So, we used the PVP, because it can dissolve in the same ethanol as the propolis was. But it has problem of the PVP is the very fast dissolve and non-stable. We solved the problem by using PVA fiber mats for the top and base to keep the examples more stable and the propolis could be diffused to the wound when the polymer dissolving.

7. THE ANTIMICROBIAL ACTIVITY

Some wounds are despite optimal treatment but it is slow heal. This is the result of the infection. So, the good wound dressing not only uses for cover the wound but must also

be effective in antibacterial as well. Pathogenic organisms causing surgical wound infections are antibiotic resistant organisms, such as methicillin resistant *Staphylococcus aureus* (MRSA).¹⁸ From the reason above, the fiber mats were tested the antibacterial and antifungal activity assays. The antibacterial activity was studied using disc diffusion method. *S. aureus*, MRSA, *P. aeruginosa*, *B. cereus* and *S. epidermidis* were used as tested microorganisms. PVA-PVP/propolis and PVA-PVP/propolis/CL-CMS fiber mats layers discs were prepared from using a paper hole-punch with the diameter of 6 mm. Each surface of fiber discs was sterilized by ultraviolet light irradiation. The microorganisms tested were grown on nutrient agar and incubated at 37 °C for 24 hrs. And inhibition zones were measured. In this studied was used the gentamicin (0.5% w/v) for the positive control.

The results of the microorganism test are shown in Tables I and II. For PVA-PVP/propolis fiber mats layers, the propolis concentration varied from 0% w/v to 5% w/v. The inhibition zones were increased with the concentration of propolis contains. The antibacterial activities of the fiber mats layers were found to depend on the concentration of propolis added.

The result of PVA-PVP/propolis/CL-CMS fiber mats layers showed that no difference of the inhibition zone. From the result, it can be seen that the antibacterial activity of sample depends on the propolis contain. Because, the increasing amount of CL-CMS does not affect the antibacterial properties.

The antibacterial activity of the sample due to the antibacterial activity of propolis could result from chemical compositions which can inhibit or kill a wide range of bacterial species. The propolis has many reports about components that are involved in antibacterial, antifungal, antioxidant and other activities,¹⁹ such as flavonoids, apigenin, chrysin, flavonones and naringenin.^{20,21} The main compound of propolis are acid such as caffeic, p-Coumaric acid, 3-prenyl-4-hydroxycinnamic, 3,5-diprenyl-4-hydroxycinnamic, 2,2-dimethyl-8-prenyl-2H-1-benzopyran-6-propenoic, kaempferol derivative.²² Cushnie et al.²³ reported about the antimicrobial activity of flavonoids by inhibition of nucleic acid synthesis, cytoplasmic membrane function or energy metabolism. For example of the antibacterial activity of propolis on *S. aureus*, the result is the effect of the propolis compounds such as triterpenic acids.^{22,24}

Table I. The inhibition zone of PVA-PVP/propolis fiber mats layers.

Propolis concentration (% w/v)	Inhibition zone (mm)				
	<i>S. aureus</i>	MRSA	<i>P. aeruginosa</i>	<i>B. cereus</i>	<i>S. epidermidis</i>
0	0	0	0	0	0
1	0	0	0	0	0
3	7.2±0.05	7.1±0.00	7.9±0.09	7.0±0.05	8.0±0.08
5	11.4±0.09	12.2±0.04	11.5±0.08	11.2±0.08	12.0±0.10

Table II. The inhibition zone of PVA-PVP/propolis/CL-CMS fiber mats layers (Fixed propolis at 5% wt/v).

CL-CMS contains (% wt/v)	Inhibition zone (mm)				
	<i>S. aureus</i>	<i>MRSA</i>	<i>P. aeruginosa</i>	<i>B. cereus</i>	<i>S. epidermidis</i>
0	11.4 ± 0.09	12.2 ± 0.04	11.5 ± 0.08	11.2 ± 0.08	12.0 ± 0.10
5	11.5 ± 0.10	12.2 ± 0.04	11.6 ± 0.04	11.4 ± 0.06	12.1 ± 0.21
10	11.4 ± 0.10	12.4 ± 0.05	11.6 ± 0.04	11.1 ± 0.06	12.2 ± 0.13
15	11.5 ± 0.05	12.0 ± 0.09	11.2 ± 0.10	11.2 ± 0.09	11.8 ± 0.08
15*	0	0	0	0	0

Note: 5* is the PVA-PVP/CL-CMS fiber mats layers (no propolis).

8. CYTOTOXICITY AND ANTIVIRAL ASSAY

Cytotoxicity of samples was investigated on Vero cells. The staining of 50,000 µg/ml of samples solution in dimethyl sulfoxide (DMSO) was serially two-fold diluted with in Eagle's minimum essential medium (MEM; Gibco, UK). Each of the samples concentration was added to quadruplet wells onto 96-well tissue culture plate. After that, 1×10^5 cells/ml of Vero cells were added and incubated at 37 °C for 72 hrs in humidified 5% CO₂ atmosphere incubator. Leaving the media and cells were stained by 0.1% crystal violet in 1% ethanol for 20 min to evaluate toxicity on Vero cells. The concentration of 50% cytotoxicity dose (CD₅₀) was expressed as a concentration of the extract that caused cell detachment from the wells by 50%. The CD₅₀ value was determined according to modified protocol from Reed and Muench (1938). The staining cells were showed in Figure 2.

Cytotoxicity was studied on the samples containing various concentration of propolis (0, 1, 3 and 5% w/v). From Table III, the result showed that the CD₅₀ decreased with increased the propolis concentration. This result is due to some effect of the propolis on the polymer to the action on the Vero cell, which may be an effect on cell deformation (Fig. 2(c)).

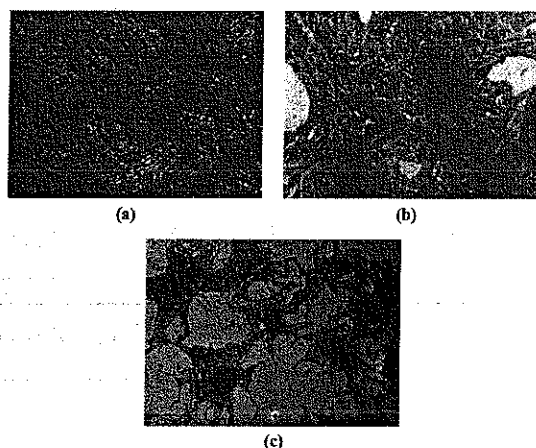


Fig. 2. Morphology of the Vero cells, (a) after test, (b) sample concentration less than CD₅₀ and (c) sample concentration more than CD₅₀.

The anti-HSV test by the Vero cells were cultured in 24-wells, each well has the volume of 500 µl. After that, the Vero cells were incubated at 37 °C in humidified 5% CO₂ atmosphere incubator for 2–3 days. Then, the media was discarded 300 µl of each well and added the 100 µl of HSV concentration of 100 plaques/0.1 ml for 1 hr at room temperature on rocking platform. After that, added the 200 µl sample at the concentration of non-toxicity on Vero cells. The medium and overlay medium were added, the volume of 200 µl and 300 µl, respectively. Then, the sample was incubated at 37 °C in humidified 5% CO₂ atmosphere incubator for 3–4 days. After that, the media was discarded and infected cells were stained with 0.1% crystal violet in 1% ethanol for 20 min. The percentage of inhibition was determined by reduction of plaque number comparing to untreated virus control.

Herpes simplex virus (HSV) is a human pathogen.

The virus is the causative agent of herpes disease. Primary infection is usually acquired in childhood and is most often asymptomatic, after which virus becomes latent in neurons of cranial nerve ganglia (HSV-1) or sacral ganglia (HSV-2).²⁵ This test chooses the sample concentration at the nontoxic on the Vero cell from the CD₅₀. The anti-HSV activity was shown in Table IV. The results showed that the cytotoxicity of the fiber mats increased with the propolis concentration, but inhibition of HSV-1F and HSV-2G showed no significant difference when compared with the sample of no propolis and sample adding the propolis at 5% w/v.

Moreover propolis in many reports showed the biological activity on antiviral activity against HIV-1,²⁶ HSV-type 1^{26,27} and HSV-type 2.²² Propolis is a resinous from various plant sources. It is a natural product which contains by many components, but the widely known is flavonoids,

Table III. Cytotoxicity of fiber mat layer with varies propolis concentration on vero cell.

Concentration of propolis in the samples (wt/v%)	CD ₅₀ (µg/ml)
0.0	794.38 ± 0.00
1.0	431.21 ± 28.64
3.0	414.67 ± 28.64
5.0	150.83 ± 42.18

Table IV. Anti-HSV of the fiber mat layers with varies propolis concentration.

Propolis concentration on samples (% w/v)	Concentration ($\mu\text{g/ml}$)	HSV-1F	HSV-2G
0	650	69.46 ± 2.06	46.86 ± 2.63
1	320	47.78 ± 6.25	39.88 ± 0.42
3	320	54.21 ± 1.91	39.99 ± 0.29
5	80	56.25 ± 2.94	40.66 ± 1.32

moronic acid derivatives and caffeic acid.²⁶ These components also showed the antiviral properties.²⁸

9. CONCLUSIONS

The fiber mats layers prepared by electrospinning technique for wound dressing application was successful. The top and base of the sample prepared by PVA electrospun fiber mats, the morphology showed smooth and beads free. The middle layer prepared by PVP added CL-CMS and propolis showed that the smooth fiber and the CL-CMS powder could be observed. The samples have the good antibacterial activity and the inhibition zone with increasing the propolis and the CL-CMS concentration. The anti-HSV activity of the sample with no propolis and adding 5% w/v of propolis were $69.46 \pm 2.06\%$ and $56.25 \pm 2.94\%$ of HSV-1F and $46.86 \pm 2.63\%$ and $40.66 \pm 1.32\%$ of HSV-2G respectively.

Acknowledgment: We acknowledge the Physics Program, Faculty of Science and Technology, Pibulsongkram Rajabhat University, Faculty of Science and Graduate School of Chiang Mai University, Office of the Higher Education Commission (OHEC) Thailand, The Thailand Research Fund (TRF) and we wish to thank the National Research University Project under Thailand's Office of the Higher Education Commission (OHEC) for their financial support. The authors would like to thank Dr. Denis Russell Sweatman for English correcting of the manuscript.

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Received: 15 April 2014. Accepted: 30 July 2014.