CHAPTER 3

Experimental Procedures

In this chapter, the experimental procedures for the preparation and characterization of electrospun fibers are described along with a detailed presentation of each technique applied.

3.1 Materials and Instruments

3.1.1 Chemical substances

- Polyvinyl pyrrolidone (PVP K90, Kollidon® 90F, MW 1,250,000 BASF, Germany)
- Polyvinyl pyrrolidone (PVP K30, Kollidon® 30, MW 50,000 BASF, Germany)
- Polyvinyl alcohol (MW 85,000 – 146,000 Sigma –Aldrich, Germany)
- Polyvinyl alcohol (MW 47,000 Sigma –Aldrich, Germany)
- Hydroxy propyl cellulose (MW 370,000 Sigma –Aldrich, Germany)
- Brazil propolis (Chiangmai Healthy Product Co., Ltd., Thailand)
- Menthol (Purchased from Union Science Co., Ltd., Thailand)
- Thymol (Ajax Finechem Ltd., Australia)
- Methyl salicylate (Purchased from Union Science Co., Ltd., Thailand)
- Eucalyptol oil (Purchased from Union Science Co., Ltd., Thailand)

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- Tween 80 (Purchased from Srichand United Dispensary Co., Ltd., Thailand)

- Absolute ethanol (Merck, Germany)

- Sterile normal saline solution (A.N.B. Laboratories CO., Ltd., Thailand)

- Dimethyl sulfoxide (Merck, Germany)

- Glutaraldehyde (Merck, Germany)

- Brain Heart Infusion (Bacto™, France)

- Agar (Helicopter, Thailand)

- Sucrose (Mitr Phol sugar, Thailand)

- Crystal violet solution (Merck, Germany)

- Folin-Ciocalteu's phenol reagent (Merck, Germany)

- Sodium carbonate (Ajax Finechem, Australia)

- Gallic acid (Sigma –Aldrich, Germany)

3.1.2 Bacterial strains

- *Streptococcus mutans* ATTC 25175

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3.1.3 Instruments

- Lyophilizer (Model Alpha 1-2 LD Christ, United Kingdom)

- High voltage power supply
- Collector
- Feeding pump

} Modified and set up at the Department of Physics and Materials Science, Chiang Mai University

- Glass syringe with a 20-gauge stainless steel needle

- Scanning electron microscope (Model JSM-5800LV Jeol, Japan)

- Scanning electron microscope (Model JSM-6335F Jeol, Japan)

- Ion sputtering (Model Balzer SCB040)

- Differential scanning calorimeter (Model Mettler Toledo851-e, Switzerland)

- FT-IR spectrometer (Model Thermo Nicolet 6700 Thermoscientific, USA)

- Powder X-ray diffractometer (Model Miniflax II, RigaKu, Japan)

- UV-vis spectrophotometer (Model UV-1800 Shimadzu, Japan)

- Analytical balance (Model GR200 A&D, Japan)

- Hot plate and magnetic stirrer (Model IKA RH basic, USA)

- Optical microscope (Olympus, Japan)

- Digital camera (Nikon, Japan)

- Conductivity meter (Model Mettler-Toledo AG 8603, Switzerland)

- Viscometer (Brookfield, DV-II, USA)

- Tensiometer (Kruss® K6, Germany)

- Anaerobic jar and Anaerocult®A (Merck, Germany)

- Vortex mixer (Model Genie2, USA)

- Centrifuge (Model HARRIER 18/80 Sanyo, Japan)

- Ultrasonic probe (Model Sonics VibraCell, USA)

- Spectrophotometer (Model Thermo Spectronic, USA)

- Hot Air oven (Model Binder, Germany)

- Autoclave (Model Tomy SS-325, Japan)

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3.2 Methods Used

3.2.1 Propolis extraction

The extraction of propolis followed the procedure referred to by Sanpa et al. [72]. Propolis, cooled with liquid nitrogen, was ground before extraction. 30 Grams of grounded propolis was mixed with 300 mL of 70% ethanol and extracted by using an ultrasonic technique for 30 minutes, and then filtered. The filtrate was evaporated by using a rotary evaporator under reduced pressure at a temperature below 40°C. The residue was lyophilized and the dry powder of propolis extract was kept in a closed container and protected from light.

The percent yield of propolis extract was calculated as the dry weight of the propolis extract gained from each extraction after the lyophilization process, and was compared with the initial weight of propolis before extraction; the percent yield was calculated as follows:

$$\text{Percent yield} = [(W_1 - W_2) / W_1] \times 100 \quad (3.1)$$

Where W_1 = Weight of propolis before extraction

W_2 = Weight of propolis after extraction

3.2.2 Determination of the phenolic compounds in the propolis extract

The polyphenols in the propolis extracts were determined by the Folin-Cioculteau method [73]. Propolis extracts were dissolved in absolute ethanol and diluted to 0.02% (w/v). 1 mL of tested solution was mixed with 0.5 mL of Folin-Cioculteau reagent and 0.5 mL of 10% (w/v) sodium carbonate. The absorbance was read at 765 nm after a one-hour incubation period in a dark condition, at room temperature. Gallic acid was used as the standard to produce the calibration curve. The total phenolic content is expressed as gallic acid equivalents (GAE) in units of mg/g. The samples from each extraction were tested in triplicate.

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3.2.3 Preparation of polymer based fibers by using the electrospinning technique

1) Preparation of polyvinyl pyrrolidone electrospun fibers

The PVP electrospun fibers were prepared by dissolving PVP in absolute ethanol. PVP K90 was studied at concentrations of 4%, 6%, 8%, 10% and 12% (w/v). PVP K 30 was studied at concentrations of 30%, 35% and 40% (w/v). The polymer solution was stirred with a magnetic stirrer at an ambient temperature, for 2 hours. The homogeneous polymer solution was poured into a glass syringe equipped with a 20-gauge stainless steel needle. A power supply was used at voltages ranging from 15-18 kV. The feeding rate was controlled by a syringe pump at 2 mL per hour. The distance between the tip of the syringe and the collector was 15 cm. The spinning process was carried out at room temperature. To study the effect of a mixed solvent of ethanol and water on PVP based electrospun fibers, ethanol and water at respective ratios of 10:0, 8:2 and 7:3 were used as solvents.

2) Preparation of polyvinyl alcohol electrospun fibers

The PVA electrospun fibers were prepared by dissolving PVA in deionized water that was heated up to 70°C and gently stirred for 3 hours to dissolve solid materials. PVA (MW 85,000-146,000) solutions with concentrations of 6%, 8% and 10% (w/v) and PVA (MW 47,000) solution with concentrations of 20%, 25% and 30% (w/v) were prepared. A power supply of 22 kV was used. The feeding rate was controlled by a syringe pump at 2 mL per hour. The distance between the tip of the syringe and the collector was 15 cm. The spinning process was carried out at room temperature. To study the effect of a mixed solvent of water and ethanol on PVA based electrospun fibers, ethanol and water at respective ratios of 10:0, 8:2, 7:3 and 5:5 were used as solvents.

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3) Preparation of hydroxypropyl cellulose electrospun fibers

The HPC electrospun fibers were prepared by dissolving HPC in deionized water and gently stirred for 2 hours to dissolve solid materials. A power supply was used at a voltage ranging from 15-25 kV. The feeding rate was controlled by a syringe pump at 2 mL per hour. The distance between the tip of the syringe and the collector was 15 cm. The spinning process was carried out at room temperature.

3.2.4 Preparation of propolis fast dissolving nanospun fibers using the electrospinning technique

1) Preparation of propolis-PVP K90 fast dissolving electrospun fibers

To ensure uniformity of propolis content in the electrospun fibers, propolis 2-5% (w/v) was first dissolved in absolute ethanol and stirred with a magnetic stirrer for 1 hour. Then, PVP K90 was added to the electrospinning solution and the solution was magnetically stirred to obtain a homogeneously dissolved solution. 10 mL of the homogeneous propolis-PVP solution was paired into a glass syringe equipped with a 20-gauge stainless steel needle. A power supply of 15 kV was used. The feeding rate was controlled by a syringe pump at 2 mL per hour. The distance between the tip of the syringe and the collector was 15 cm. The spinning process was carried out at room temperature.

2) Formulation of propolis fast dissolving nanospun fibers as an oral strip using the electrospinning technique

Flavoring agents such as menthol, thymol, methyl salicylate and eucalyptol oil were added to a propolis-PVP electrospinning solution. Tween 80 as wetting agent was added for improved wettability of propolis-PVP electrospun fibers.

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3) Preparation of chlorhexidine-PVP K90 fast dissolving electrospun fibers

20% (w/v) of chlorhexidine stock solution was added to an 8% (w/v) PVP K90 polymer solution to make 4.8% (w/v) of chlorhexidine in the spinning solution. A fixed electrical potential of 15 kV, a feed rate at 2 mL per hour and a distance of 15 cm between tip and the collector were applied for the electrospinning process.

3.2.5 Characterization of physical properties of electrospinning solutions

1) Viscosity

The viscosities of electrospinning solutions were measured by using a Brookfield, DV-II viscometer and were displayed in units of cP.

2) Surface tension

The surface tensions of electrospinning solutions were measured by using a ring method with a DuNouy tensiometer.

3) Conductivity

The conductivity of electrospinning solutions was measured by using a conductivity meter.

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3.2.6 Characterization of physical properties of electrospun fibers

1) Morphology

A scanning electron microscope (FIB Quanta 200 3D) was used to investigate the morphology of the electrospun fibers. Samples were first gold sputter-coated and pictures were taken with a scanning electron microscope. The diameters of electrospun fibers were measured at more than 50 points from SEM images by using Image J software. Average diameters and standard deviations were calculated.

2) FTIR spectroscopy

Fourier-transform infrared (FTIR) spectra were obtained on a Thermo Nicolet 6700 Thermoscientific (USA) as KBr pellets. The scanning range was 750 – 4000 cm^{-1}.

3) X-ray diffraction analysis

The X-ray diffractograms were obtained using a Miniflax II X-ray diffractometer, RigaKu (Japan), in the 2θ range of 10-60°.

4) DSC analysis

Differential scanning calorimetric (DSC) analyses were obtained using a Mettler Toledo 851-e (Switzerland) for thermal analysis of the electrospun fibers.

3.2.7 Wetting and disintegration/dissolution properties of electrospun fibers

The test used followed the procedure as described in the literature [40,42]. Two layers of absorbent paper were placed in a petri dish with a diameter of 10 cm and wetted with distilled water; excess water was drained away. The electrospun fibers were placed on wet paper and observed during the time that electrospun fibers were completely wetted or dissolved in water on wet paper.

The wetting and disintegration/dissolution processes were recorded at 120 and 400 frames per second with a digital video recorder (Nikon, Japan).

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3.2.8 Determination content of propolis in electrospun fibers

The content of propolis incorporated in electrospun fibers was determined by using an UV-vis spectrophotometer. The samples of propolis-PVP electrospun fibers were weighted accurately, and dissolved in absolute ethanol. The sample solutions were analyzed at a wavelength of 300 nm using a UV-1800 Shimadzu UV spectrophotometer (Japan). The amount of

propolis present in the electrospun fibers was calculated using a calibration curve derived from propolis extract in absolute ethanol. All measurements were conducted in sextuplet and the results were reported in terms of average value and standard deviation.

3.2.9 Antimicrobial activities of propolis-PVP electrospun fibers

1) Preparation of bacterial suspension

A stock of S. *mutans* ATCC 25175 was prepared in glycerol and kept at minus 20°C for further use. *S. mutans* was inoculated into brain heart infusion (BHI) broth and incubated for 18-24 hours at 37°C in an anaerobical condition. In this study, the bacterial suspension used was adjusted and turbidity with a standard 0.5 McFarland solution was compared, which is equivalent to $1x10^8$ CFU/mL.

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

The minimum inhibitory concentration (MIC) was determined as the lowest concentration of the extract that inhibited the growth of the microorganisms. The minimum bactericidal concentration (MBC) was defined as the lowest concentration of the extract that killed the bacteria at a 99.9% rate. In this study, a two-fold dilution method to determine the MIC and MBC was used. The microorganisms were tested on serial dilutions of propolis extract that was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 300 mg/mL and compared, for positive control, with a chlorhexidine mouthwash solution starting at a concentration of 1 mg/mL. Then, the culture was incubated for 24 hours in an anaerobical condition at a temperature of 37°C. The minimum concentration of propolis extract that did not show turbidity of the broth was determined as MIC. The MBC was determined by streak plating the tested solutions from the tubes, which did not show any growth after incubation on BHI agar, and incubated for 24 hours in the same

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conditions. The MBC was determined as the lowest concentration of propolis extract, which inhibited growth of microorganisms by 99.9%.

3) Antibacterial activity of propolis fast dissolving electrospun fibers

The antibacterial activity of mouth-dissolved electrospun fibers against *S. mutans* was determined by the agar diffusion method. A bacteria culture was swabbed on the presence of BHI agar. The electrospun fibers were dissolved in 1 mL of sterile distilled water or DMSO to a final concentration equivalent to 1, 2.5, 5, 7.5, 10 and 15 MIC using a vortex mixer. The sterile filter paper discs (6 mm in diameter) were immerged into these samples and then placed on the BHI agar. The plates were then incubated at 37°C for 24 hours, anaerobically. All tests were performed in triplicate and diameters of the inhibited clear zones were measured after the incubation.

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

The bacterial adherence to a glass surface based on a method developed by Rahim and Khan [74] was studied. A bacteria culture of 200 µL was grown in 3 mL of BHI broth containing 1% sucrose (w/v) in a test tube containing *S. mutans* at sub-MIC concentrations (0.015 MIC to 1 MIC) of the propolis from propolis extract or propolis electrospun fibers. After an incubation period of 18 hours at 37°C at surface in an angle of 30°, the adhering bacteria were washed away with a normal saline solution and re-suspended in a normal saline solution using an ultrasonic probe. The amount of adherent bacteria was determined by measuring absorbance at OD_{550nm} and calculated as the percentage of adherence. Sterile distilled water was used as a control of which the total bacteria number was known and represented 100% adhesion or was replaced with a sample of mouthwash solutions obtained from the market. All tests were performed in triplicate. Results for this test were given as percentages of adherence, applying the following formula:

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Percentage of adherence = (OD assay / OD control) x 100 (3.2)

To investigate the amount of vital bacteria cells that adhered to the glass surface, 100 µl of the adhering bacteria was suspended in sterile normal saline (before using an ultrasonic probe) and was mixed with 900 µl of sterile normal saline and diluted to determine a suitable dilution ratio to count the colony forming units (CFUs) on an agar plate. Aliquots (100 µl) of the dilution were spread on the BHI agar plates and incubated for 24 hours at 37 °C in an anaerobical condition. The colonies were then counted at the end of the incubation period. All quantitative cultures were run in triplicate. The results were compared with the CFUs at the starting time (T_0) when *S. mutans* was contacted with the tested solution and after 18 hours (T_{18}) of the incubation period from the study on bacterial adherence to a glass surface.

3.2.11 Morphology of adherence bacteria and biofilm structure

In line with testing the inhibition of adherence of growing cells to a glass surface, 2 tubes for sample testing were added to a small piece of the glass slide (1 x 1 cm) in the broth and used for light microscopy (LM) and scanning electron microscope (SEM) studies to explore the adhering of *S. mutans* and biofilm forming on the glass surfaces. One of the glass slides was prepared, dried in room temperature and fixed with heat. A crystal violet single stain was performed for 1 minute and washed with water. Photographs were obtained using an optical microscope (Olympus, Japan) with a camera. Another glass slide was prepared for scanning by an electron microscope by immersing it in a 0.1% glutaraldehyde solution for 5 minutes, then it was washed three times with a normal saline solution and immersed in ethanol series (50, 60, 70, 90, 95 and 100%) for 20 minutes, at room temperature. The glass slides were dried and coated with gold using a sputtering coater technique. Micrographs were obtained with a scanning electron microscope [75].

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3.2.12 Stability test

Samples of the electrospun fibers were kept in aluminum pouches and kept in a desiccator through the stability test period. The morphology and physical properties of electrospun fibers, content of propolis in electrospun fibers, antimicrobial properties and inhibition of adherence of *S. mutans* to a glass surface by propolis-PVP electrospun fibers were tested using the same procedure.

3.2.13 Statistical analysis

A statistical analysis was carried out by applying a one-way analysis of variance (ANOVA) and a t-test to compare the means of the tests ($p < 0.05$ was considered significant).

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