CHAPTER II

MATHODS

1. Preparation of Polysaccharide Gel from Fruit-Hulls of Durian

Fresh fruit-hulls of durian cv. 'Monthong' (Imsabai, Ketsa, and Doorn, 2002) were collected and cleaned. The durian fruit-hull or pericarp was blended. One kilogram of blended fresh durian fruit-hulls was dried by hot air oven at 70 °C for 48 hrs, about 200 grams of dried durian fruit-hulls were obtained. Dried fruit-hulls of durian were kept in cold room (4 °C) until use. Hot acidic water extraction of polysaccharide gel from fruit-hulls was carried out and purified by modifying the method of Pongsamart and Panmaung (1998).

2. Determination of Physical Properties of Polysaccharide Gel (PG)

2.1 Viscosity of PG Aqueous Solution and Mixture of PG with Plasticizer

PG at 2% concentration in distilled water and a mixture of 2% PG with plasticizer, propylene glycol 15% w/w based on PG, were prepared. The viscosities of 2% PG and 2% PG with plasticizer mixture were measured each of 3 times, respectively by using viscosmeter. The mean values of viscosity between mixture of PG and PG with plasticizer mixture were compared.

2.2 pH of PG Aqueous Solution and Mixture of PG with Plasticizer

Two percentages of PG aqueous solution and a mixture 2% PG with 15% w/w propylene glycol based on PG were prepared and measured pH by using pH meter. The mean values of three measurements of each mixture were compared.

3. Preparation of PG Dressing Films and PG Dressing Gel

3.1 Preparation of PG Dressing Films

The PG dressing films were prepared by casting/solvent evaporation method (Remunanlopez and Bodmeier, 1996). The formulation of PG dressing film composed of 2% PG. PG powder 2 g was dissolved in 80 ml deionized water at room temperature and 0.3 g propylene glycol was added as a plasticizer, deionized water was added to make 100 ml (Gerddit et al., 2002). The solution was warmed and stirred until homogenously mixed and degased by using sonicator for 30 mins. The 55.91 g of PG solutions were spreaded onto a plastic casting area 10.2 x 24.8 cm^2 to make a solid content of PG at 4.42 mg/cm^2. The films were dried at room temperature for 6 hrs and in hot air oven at 50°C for 8 hrs. The PG dressing film was cut into pieces of 3x3 cm^2 and kept in a desiccator, and the film was sterilized by autoclave at 121°C, 15 lb/in^2 for 15 min before use.

3.2 Preparation of PG Dressing Gel

The PG dressing gel was prepared, all ingredients were sterilized previously use. The gel dressing formula composed of 93 ml of 2.5% PG in water; 5 ml propylene glycol; 1 ml of 0.56% $CaCl_2$ saturated solution and 1 ml paraben solution. Slowly mixed and added a drop by drop of saturated $CaCl_2$ solution with continuous stirred. The PG gel was mixed homogenously by using the homogenizer. The PG gel dressing was degassed by using a sonicator for 30 min and kept in a sterile glass bottle. The PG gel dressing was sterilized under UV light, wavelength 254 nm in the laminar air flow for 1 hr (Mehta and Hawxby, 2002).

4. Analysis of Physical Properties of PG Dressing Films

4.1 Thickness of PG Dressing Films

The thickness of PG dressing films were measured by the micrometer at different five points on each film. The mean values of thickness were calculated.

4.2 Mechanical Properties of PG Dressing Films

Mechanical properties of dry PG dressing films were analyzed by a universal tensile testing instrument such as tensometer equipped with 0.01 KN tension load cell. A high expansion as 50 mm. In this study, the PG dressing films were separated into 3 samples.

Sample 1, The nonsterilized PG film and PG dressing film (with plasticizer).

Sample 2, The sterilized PG film and PG dressing film (with plasticizer). The films were sterilized by using hot air oven at 120°C for 1 hrs.

Sample 3, The sterilized PG film and PG dressing film (with plasticizer). The films were sterilized in autoclave at 121°C, 15 lb/in^2 for 15 min.

The mechanical properties of PG dressing films in each sample were evaluated by the following procedure and kept in the desiccator for 24 hrs. until use. PG dressing films were cut into a small square of 2x2 cm^2 pieces. The film was carefully clamped by the upper and lower pneumatic flat-faced grips and extended by the test machine at speed of 10 mm/min until the film was certainly separated. The breaking force displayed in digital was recorded, the values of young's modulus (megapascals), stress at break, % strain at break and toughness. The mean values of five measurements in each group were compared.

4.3 Swelling Property of PG Dressing Film

The swelling of PG dressing film was determined by using the preparation of PG dressing films with 15% plasticizer based on PG weight, size 1x1 cm^2. The films were impregnated in deionized water in a preti-dish. The dimension of the films were measured every 1 hr for 24 hrs. The averages of three measurements were plotted vs time (hours).

5. Application of PG Dressings for Treatment of Wound

5.1 Animals Preparation

Eight to nine young female, cross-bred pigs (Duroc x Large White x Landrace), weighing 18 to 20 kgs, ranging in age from six to eight weeks, were used in this study. The animals were kept separately in each cage and fed a standard swine diet (*ad libitum*), as illustrated in Figure 4A. All pigs were acclimatized for at least 7 days before the experiment started.

5.2 Full-Thickness Open Wound Operation and Treatment Designation

On experiment day (day 0), before wounding operation the animals were sedated by intramuscular injection of 4 mg/kg azaperone, anesthetized with 20 mg/kg pentobarbital sodium and 6 mg/kg of analgesic drug buprenorphine by intravenous injection. All dorsal (back) skin of the pigs were shaved (Figure 4B) and surgically prepared by 10% povidone iodine and 90% alcohol solution (Figure 4C). Five to six full-thickness skin incision wounds of 2.45 cm in diameter were created with a steriled template, as illustrated in Figure 4D. The wounds were operated with scalpel and removed from the underlying subcutaneous tissue in each pig (approximately depth 7 mm), as demonstrated in Figure 4E and Figure 4F. A total of 50 replicate wounds were randomly treated by 4 treatments as follows :

Control group, the wounds were treated with 1 % povidone iodine applied on wounds which is a conventional therapy (16 total wounds).

Treatment group 1 (T_1), the wounds were treated with sterile PG dressing film size 3x3 cm^2 covered the wounds (12 total wounds).

Treatment group 2 (T_2), the wounds were treated with 1% povidone iodine and covered with sterile PG dressing film size 3x3 cm^2 (12 total wounds).

Treatment group 3 (T_3), the wounds were treated with 1 g of sterile PG dressing gel applied on wounds (10 total wounds).

All surface wounds were covered with a sterile gauze dressing and fixed to the intact skin near the edges by suturing with 2/0 nylon and 0 silk, as demonstrated in Figure 4G. To protect the dressing, the pigs were wrapped around the bodies with elastic cloth, as shown in Figure 4H. Only one dose of 4 mg/kg body weight, intramuscular injection of long acting amoxicillin was administered to the pigs as final treatment before returned the animals to their cages.

5.3 Measurement of Wound Areas and Treatment of Wound

Every 3 postoperative days (POD) on day 3, 6, 9, 12 and 15, the animals were sedated and anesthetized by the same procedure. All wounds in each treatment were opened, old dressings were removed, wound appearance were examined, and the areas of wound were measured by tracing the wound boundaries using sterile transparent sheets with permanent marker. Color photographs of the wounds were taken by digital camera. Wound were cleaned with sterile normal saline and repeated the same treatment with 1 % povidone iodine, PG dressing film, 1% povidone iodine and covered with PG dressing film, and PG dressing gel, respectively. The wounds were covered with a new sterile gauze on the top and fixed the skin near the edges by suturing with 2/0 and 0 silk nylon and were protected by using elastic cloth.

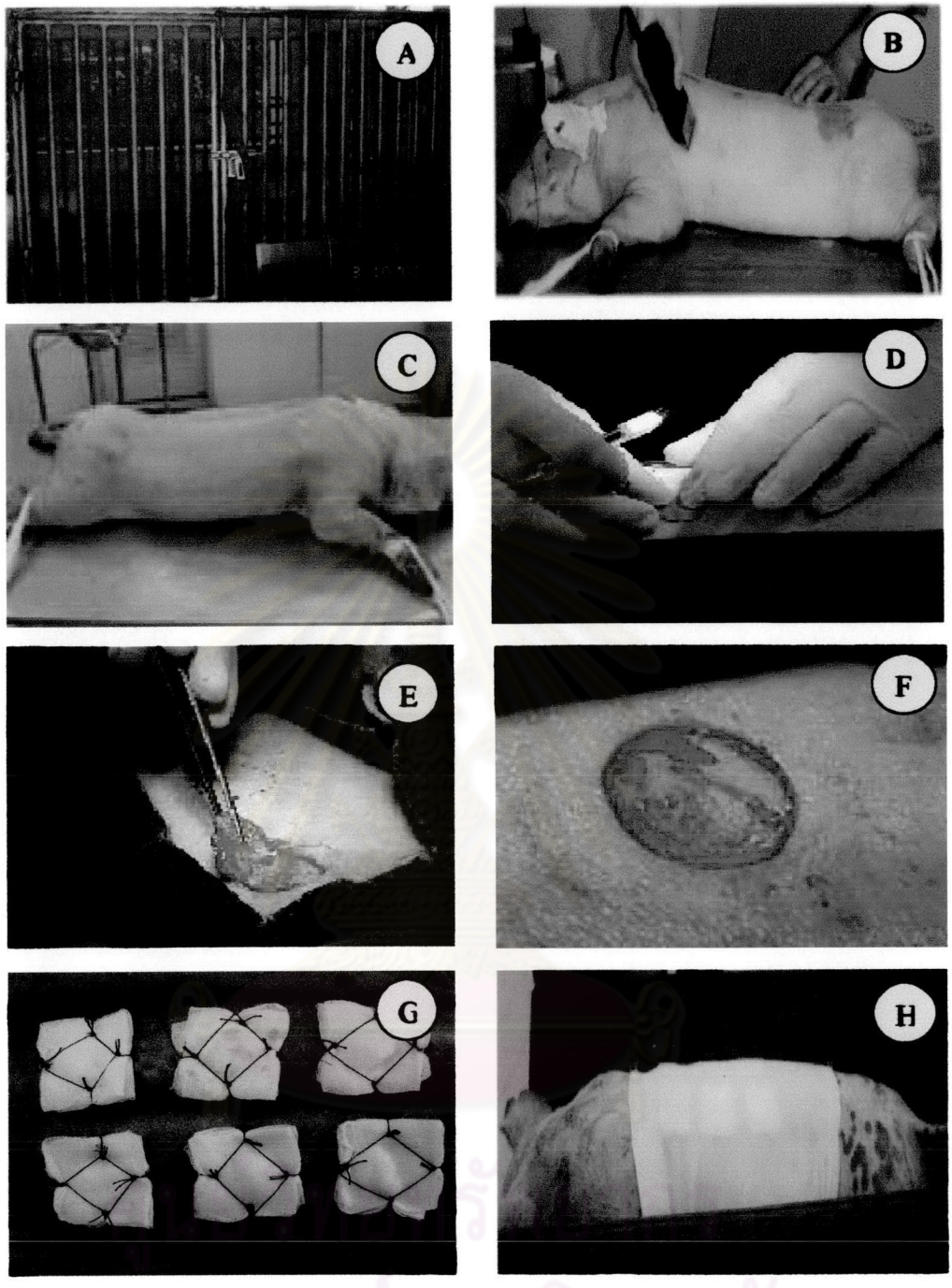

Figure 4. Wound operation and treatment. A) The animal cage for keep each pig B) Dorsal skin of the pig was shaved. C) Dorsal skin of the pig was surgically prepared. D) Create a circular drawing on the pig skin by using a 2.45 cm in diameter of a sterile template. E) The skin was cut and removed. F) A full-thickness wound size 2.45 cm in diameter. G) Wounds were covered with sterile gauze dressing and fixed to the intact skin near the edges by suturing with 2/0 and 0 silk nylon. H) The dressing and the wound sites were protected by elastic cloth.

At the end of experiment on POD 18, the animals were euthanized by intravenous injection with overdose of pentobarbital sodium. An area of one square inch of the skin of each operative site was removed deep into the epidermal and dermal layers from wound margin. The tissues were fixed in 10% neutral buffered formalin to preserve the intracellular and extracellular components, until use.

The operation of the experimental animals was approved by the Ethics Committee of Faculty of Pharmaceutical Sciences, Chulalongkorn University.

6. Evaluation of Wound Healing

6.1 Gross Pathology Evaluation

6.1.1 Wound Lesion

Gross lesion of wounds were clinically examined every 3 POD on day 3, 6, 9, 12 , 15 and 18. After opened and removed old dressings, the wounds were examined in the following criteria such as wound bed, color, exudate, swelling of wound surface and the consistency of surrounding wound tissue.

6.1.2 Wound Area

The transparent sheet with the tracing of wound area was scanned and the areas of wound size were calculated using computer program of image analysis program provided by Department of Computer Engineering, Faculty of Engineering, Chulalongkorn University.

6.2 Histopathological Evaluation

6.2.1 Preparation of Histologic Section (Luna, 1968)

6.2.1.1 Tissue Preparation

The skin tissue size 1x1 in^2 was cut into small pieces, size 2 mm x 2 cm, one sample was cut into four pieces and kept in a plastic classette box. The tissues were preserved in the fresh fixative aqueous 10%, neutral buffered solution of formaldehyde for at least 24 hrs.

6.2.1.2 Tissue Processing

After fixation, the specimens were washed with running tab water for 10-20 min. All specimens were prepared by transferred to automatic tissue processor. The procedure was monitoring by the followings process listed in Table 4.

Dehydration, the specimens were dehydrated by transferred through a series of ethyl alcohol of increasing concentrations using 80% ethyl alcohol for 2 hrs; 95% ethyl alcohol for 1 hr, 2 times; and 100% ethyl alcohol for 1 hr, 3 times, respectively.

Clearing, the specimens were transferred to clearing agent such as xylene, which is miscible with both 100% ethyl alcohol and paraffin. The specimens were infiltrated in xylene three times for 1 hr, 2 hrs and 2 hrs, respectively.

Infiltration, following the replacement of alcohol by clearing reagent, the tissues were immersed in melted paraffin (60°C), which infiltrates the tissues for 1 hr, 2 times and 2 hrs, 1 time, respectively.

Embedding, when infiltration was completed, the specimens were transferred to a fresh paraffin and embedded in a cubical paraffin mold. After cooling, the melted paraffin was hardened. The paraffin block was removed and excess paraffin was trimmed away.

Table 4. The procedure for tissue processing in automatic tissue processor.

Objective	Number	Reagent	Time (hrs.)
Dehydration	1	80% ethyl alcohol	2
	2	95% ethyl alcohol	1
	3	95% ethyl alcohol	1
	4	100% ethyl alcohol	1
	5	100% ethyl alcohol	1
	6	100% ethyl alcohol	1
Clearing	7	xylene	1
	8	xylene	2
	9	xylene	2
Infiltration	10	paraffin	1
	11	paraffin	1
	12	paraffin	2

6.2.1.3 Slide Preparation

A slide of section was prepared by the following steps: the block of paraffin was secured to the microtome and oriented appropriately with respect to the knife. With each revolution of the microtome handle, the specimen moved through the blade and a section of the desired 5 μm thickness was produced. Each successive section adhered to the proceeding one, forming a continuous ribbon. Subsequently, one or more sections were carefully separated from the ribbon and transferred to the surface of warm water in a water bath at 40-45 °C to produce softness of paraffin and flatness of the section as well as eliminating wrinkles. The flattened section was floated onto a slide, which was left for air drying in room temperature. As the preparation dried, the section adhered to the surface of the slide (Bacha and Wood, 1990).

6.2.1.4 Hematoxylin and Eosin (H&E) Staining

After the section on the slide was dried. The paraffin was removed with xylene for 10 mins, the same procedure was repeated. The specimen was rehydrated by passing through a gradual series of decreasing concentrations at 100%, 95% and 70% ethyl alcohol, respectively, for 2 min with each alcohol concentration. The specimen was washed in running tap water for 5 mins and stained with Harris hematoxylin solution for 5 mins. The section was a bluish-violet color and washed in running tap water for 5 mins. The section was removed the excess hematoxylin in 1% acid alcohol 1 dip, and washed the excess acid in running tab water for 5 mins. The section was then neutralized by dipping into saturated lithium carbonate for 4 dips and washed in running tab water for 5 mins. Counterstain the section with eosin working solution for 45 secs to produce a pink or red color. After stained, the specimen was dehydrated by passing through a gradual series of increasing concentrations of 95% ethyl alcohol 3 dips and 100% ethyl alcohol twice for 2 mins of each. The specimen was cleared (made transparent) with xylene twice for 5 mims. Permanent mounting prepared by covered the specimen with a resinous mounting medium (DPX solution) and topped with a cover slip. The process of H&E staining was illustrated in Figure 5 (Luna, 1968).

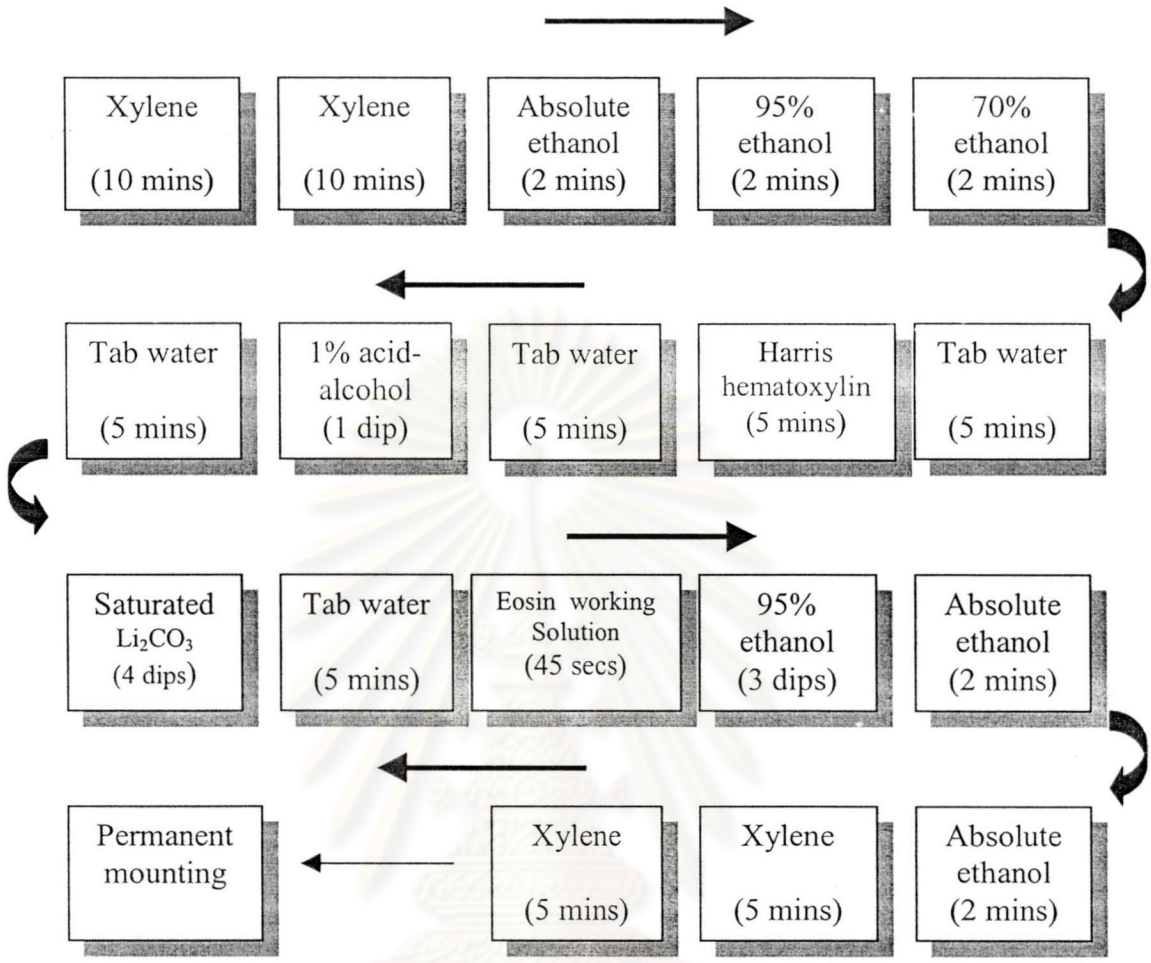

Figure 5. Hematoxylin and Eosin (H&E) staining chart.

6.2.2 Histopathological Analysis

Normal histology of the skin composed of three layers as the epidermis, dermis and hypodermis. The criteria of histopathological lesions were evaluated followings the skin layer as;

1. *Epidermal hyperkeratosis and epidermal hyperplasia* were determined by proliferation of epithelium with increased keratin production in stratum corneum layer.

2. *Dermatitis* was examined continuously 2 stages of inflammation such as
   - *2.1 Subacute dermatitis* was characterized by a number of PMNs and some macrophages aggregated from upper to lower dermis, followed increased vascularization with some congestion and hemorrhage.
   - *2.2 Chronic dermatitis* was characterized by vascularization with chronic inflammatory cells (lymphocytes and plasma cells) aggregation in the dermal layer and multifocal dermal fibrosis.

3. *Dermal fibrosis* was determined by an increasing a number of reactive fibroblasts with increased collagen fiber in the dermal layer. Including the arrangement of reactive fibroblast.

4. *Dermal granuloma* was characterized by central necrosis and proliferative zone surrounded by macrophages, lymphocytes and plasma cells with occasionally foreign-body giant cells throughout the deep layer of dermis.

The lesions were given a score ranging from 0 (no remarkable lesions), 1 (mild), 2 (moderate) and 3 (severe).

6.3 Statistical of Histopathological Analysis

Statistical significant of mean values of wound area was determined at $p < 0.05$ using one-way analysis of variance (ANOVA) : equal variances assumed using LSD.

The values of the histopathological scores were averaged and expressed as the mean and standard error of mean. All statistical evaluations were performed by one-way analysis of variance (ANOVA). The results were considered significant at $p < 0.05$.