CLINICAL EFFICACY OF BIOCELLULOSE WOUND DRESSING CONTAINING SILK SERICIN AND POLYHEXAMETHYLENE BIGUANIDE FOR SPLIT-THICKNESS SKIN GRAFT DONOR SITES

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ประสิทธิศักย์ทางคลินิกของสิ่งตกแต่งแผลไบโอเซลลูโลสผสมเซริซินจากไหมและโพลีเฮกซะเมธิลีน ไบกัวไนด์ในการรักษาบาดแผลที่ถูกตัดผิวหนังบางส่วนเพื่อปลูกถ่าย

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สิ่งตกแต่งแผลไบโอเซลลูโลสประกอบด้วยเซริซินจากไหมซึ่งมีคุณสมบัติในการเร่งกระบวนการหายของบาดแผล และโพลีเฮกซะเมธิลีน ไบกัวไนด์ เป็นสารฆ่าเชื้อที่มีประสิทธิภาพและความปลอดภัยในการรักษาบาดแผล ในการศึกษานี้เป็นการศึกษาแบบไปข้างหน้า โดยจับคู่เปรียบเทียบแบบสุ่มและปิดบังทางเดียว เพื่อประเมินความปลอดภัยและประสิทธิศักย์ทางคลินิกของสิ่งตกแต่งแผลไบโอเซลลูโลสผสมเซริซินจากไหมและโพลีเฮกซะเมธิลีน ไบกัวไนด์ เปรียบเทียบกับแผ่นปิดแผลทางการค้า (แบคทิกราส) ในอาสาสมัครสุขภาพดี (การวิจัยทดลองทางคลินิกขั้นที่ 1) และในการรักษาบาดแผลที่ถูกตัดผิวหนังบางส่วนเพื่อปลูกถ่าย (การวิจัยทดลองทางคลินิกขั้นที่ 2) โดยมีอาสาสมัครสุขภาพดีทั้งหมด 105 คน เข้าร่วมในการศึกษาวิจัยทางคลินิกขั้นที่ 1 ผลการศึกษาพบว่าค่าความแดงและความดำของผิวหนังที่ปิดด้วยสิ่งตกแต่งแผลไบโอเซลลูโลสและ แผ่นปิดแผลแบคทิกราส ไม่แตกต่างกันอย่างมีนัยสำคัญ ส่วนการศึกษาประสิทธิศักย์ทางคลินิกในผู้ป่วย 21 ราย (32 บาดแผล) พบว่าระยะเวลาการหายของบาดแผลที่ปิดด้วยแผ่นปิดแผลทั้ง 2 ชนิด ไม่แตกต่างกันอย่างมีนัยสำคัญ แต่ค่าความแดง ค่าความดำ และค่าการสูญเสียน้ำจากผิวของแผลที่รักษาด้วยสิ่งตกแต่งแผลไบโอเซลลูโลสต่ำกว่า แผ่นปิดแผลแบคทิกราส อย่างมีนัยสำคัญทางสถิติ ไม่พบอาการหรืออาการแสดงของการติดเชื้อที่บาดแผล หรือเหตุการณ์ไม่พึงประสงค์จากการใช้แผ่นปิดแผลทั้ง 2 ชนิด คะแนนความเจ็บปวดของบาดแผลที่ปิดด้วยสิ่งตกแต่งแผลไบโอเซลลูโลสต่ำกว่าแผ่นปิดแผลแบคทิกราสอย่างมีนัยสำคัญทางสถิติ ที่ 1 ถึง 5 วัน โดยสรุปสิ่งตกแต่งแผลไบโอเซลลูโลสผสมเซริซินจากไหมและโพลีเฮกซะเมธิลีน ไบกัวไนด์มีความปลอดภัยและมีประโยชน์ต่อการเพิ่มคุณภาพของแผลเป็น ลดความเจ็บปวดของบาดแผล ป้องกันการติดเชื้อ โดยไม่ก่อให้เกิดเหตุการณ์ไม่พึงประสงค์ เหมาะสำหรับใช้เป็นแผ่นปิดแผลทางเลือกในการรักษาบาดแผลที่ถูกตัดผิวหนังบางส่วนเพื่อปลูกถ่าย

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SUPAMAS NAPAVICHAYANUN: CLINICAL EFFICACY OF BIOCELLULOSE WOUND DRESSING CONTAINING SILK SERICIN AND POLYHEXAMETHYLENE BIGUANIDE FOR SPLIT-THICKNESS SKIN GRAFT DONOR SITES. ADVISOR: PROF. PORNANONG ARAMWIT, Ph.D., CO-ADVISOR: ASSOC. PROF. APICHAI ANGSPATT, M.D., 290 pp.

A biocellulose wound dressing composed of silk sericin as an accelerative wound healing component and polyhexamethylene biguanide (PHMB) as an antimicrobial agent which showed effective and safe wound treatment. A prospective, single-blinded, randomized controlled matched-pair study was designed to evaluate the safety and the clinical efficacy of the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras® in healthy volunteers (phase I clinical study) and in split-thickness skin graft (STSG) donor site wound treatment (phase II clinical study). There were 105 healthy volunteers in phase I clinical study. The results showed that the erythema and melanin levels of skin covered with both dressings were not significantly different. In the phase II clinical study, 21 patients with 32 STSG donor site wounds were included in the study. The wound healing time was not significantly different between dressings. The melanin levels, erythema levels, and TEWL levels of the group treated with the biocellulose wound dressing were significantly lower than the control group. No signs of infection and adverse effect were observed in wounds covered with either dressing. The pain scores of wounds covered with the biocellulose wound dressing were significantly lower than Bactigras® at days 1 to 5. In conclusion, the biocellulose wound dressing containing silk sericin and PHMB exhibits safety and many benefits including high scar quality, pain reduction, infection protection without adverse events. It is appropriate for use as an alternative treatment for STSG donor site wounds.

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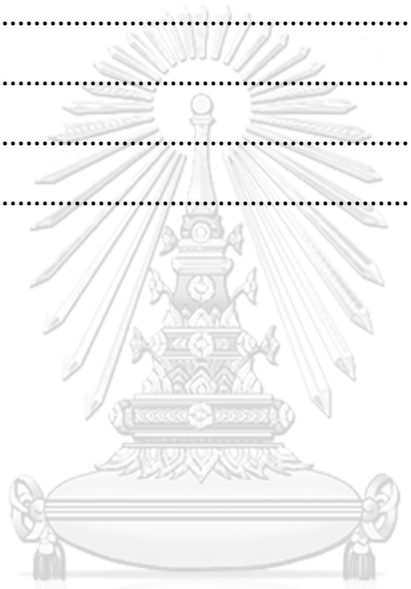

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List of abbreviations

ALP	=	Alkaline phosphatase
ALT	=	Alanine aminotransferase
AST	=	Aspartate aminotransferase
BMI	=	Body mass index
BUN	=	Blood urea nitrogen
Cr	=	Creatinine
ECM	=	Extracellular matrix
EGF	=	Epidermal growth factor
DNA	=	Deoxyribonucleic acid
FGF	=	Fibroblast growth factor
FTSG	=	Full-thickness skin graft
GAGs	=	Glycosaminoglycans
IL	=	Interleukin
IGF-1	=	Insulin-like growth factor 1
LDI	=	Laser doppler imaging
PDGF	=	Platelet-derived growth factor
PHMB	=	Polyhexamethylene biguanide
PGs	=	Proteoglycans (PGs)
RNA	=	Ribonucleic acid
STSG	=	Split-thickness skin graft
TEWL	=	Transepidermal water loss
TGF	=	Transforming growth factor

TNF	=	Tumor necrosis factor
VAS	=	Visual analogue scale
VVS	=	Vancouver scar scale
WBC	=	White blood cells

CHAPTER 1 INTRODUCTION

1.1 Background and Rationale

A split-thickness skin graft (STSG) donor site wound is a wound that involves the epidermis and some part of the dermis because of skin detachment by instrument [1]. This area is painful, red, and swollen. It usually recovers in around 7–14 days [2]. The healing rate may be slow depending on the wound environment. A good wound environment, clean and with optimal moisture, leads to a short healing time. However, it may take a long time, up to a month, if a poor environment and infection result in a chronic wound. The goals of STSG donor site wound treatment are to accelerate wound healing, prevent infection, reduce pain, and maintain an optimal environment for healing promotion [3]. Dressings with healing acceleration agents and antimicrobial agents are very important in helping to achieve the goals of treatment. They are usually applied to the wound based on exudate level and infection risk [4]. Many dressings have been developed for the treatment of STSG donor sites. Moist wound dressings seem to have more advantages for treatment than non-moist wound dressings [3]. However, they are very expensive, which leads to a lack of their use in Thailand. Moreover, combination of a wound healing acceleration agent and an antimicrobial agent in the dressing has not been developed domestically. In Thailand, wound dressings that can activate collagen synthesis for faster healing are not currently used. The only wound dressings used today are imported at high cost. Therefore, most patients cannot afford the treatment and miss the opportunity to be treated with technologically advanced products. This study will investigate a new choice for STSG donor site wound treatment that could promote wound healing and prevent infection.

Biocellulose is produced by *Acetobacter xylinum* in ripe coconut water which is normally a waste product that is discarded into the environment and result in pollution. It has an ultrafine fibrous structure that can hold a large amount of water, more than 200 times its dry weight [5]. Because of the water-holding capacity of biocellulose, it can retain moisture and is a good environment

for wound healing. It has a nanoporous structure that can transfer medication to the wound while maintaining a proper barrier for wound protection. Ultrafine networks of biocellulose are dense so they are barriers for cell migration into material, which leads to pain reduction [6]. Moreover, it has a cooling effect [7] without allergic reaction or irritation [8]. Biocellulose has many advantages for wound healing such as transparency, autolytic debridement, acceleration of re-epithelialization, and fewer daily wound dressing changes [5]. Because of many benefits of biocellulose, it is used in many applications including cosmetics and medical devices such as wound treatment dressing material.

Silk sericin is a protein from silk cocoons which can accelerate the proliferation of fibroblast cells and activate collagen synthesis. Collagen is one of the important parts in the wound healing process. It is synthesized from fibroblast cells under the skin. Collagen is migrated into the wound and accelerates wound healing [9]. Silk sericin can promote wound healing and is a biocompatible agent. It can promote smooth treatment for skin defects, increase skin elasticity, and has an anti-aging effect. A previous study showed that it can activate the growth of fibroblast cells and promote collagen production without toxicity [10]. Silk sericin has been shown to reduce wound size without causing inflammation [11, 12]. In humans, silk sericin dressings accelerate wound healing without any sign of irritation [13]. Because of the properties of silk sericin, it is used in many applications including medical and pharmaceutical materials such as lotions, creams, ointments, gels, dressings, and bandages [14].

Infection is an important concern for wound care. It is a cause of delayed wound healing, morbidity, and mortality [15]. Protection from infection is a primary concern from the initiation of wound treatment. The organisms which are found in wounds are both Gram-positive and negative bacteria such as *Staphylococcus aureus*, methicillin-resistant *Staphylococcus aureus* (MRSA), and *Pseudomonas aeruginosa* [16, 17]. Therefore, topical antimicrobial agents are very important for wound treatment. There are many antimicrobial agents that are used to treat wound infections. However, most have an effect on normal cells that leads to destruction of cells and delayed wound healing. Moreover, antimicrobial agents are usually causes of pain and discomfort. There have been many reports

about microorganism resistance to antimicrobial agents. Due to these limitations, finding other antimicrobial agents for wound care that have high efficacy and fewer adverse effects is of interest. Polyhexamethylene biguanide (PHMB) is a broad-spectrum antimicrobial agent with high efficacy and low toxicity [18]. It is a biocide against aerobic and non-aerobic bacteria. Binding between the negatively charged phosphate head group of bacterial cell wall phospholipids and the cationic group of PHMB interferes with the bacterial cell membrane and leads to cell death [19]. However, this interaction is rarely found in human and animal cells. Moreover, PHMB can reduce biofilm in the wound and promote granulation tissue growth for healing without irritation [20]. It reduces local wound infection more rapidly and better than silver dressings [21]. For skin irritation, concentrations of PHMB more than 5% are harmful in rats [22] and more than 1% are unsafe in humans. However, the general concentration used in wound dressing is around 0.3% PHMB, which is safe for wound treatment [20]. Moreover, no development of bacterial resistance to PHMB has been reported [23].

Therefore, the combination of silk sericin and PHMB in biocellulose dressings would benefit STSG donor site wound treatment, because it can protect the wound from the outer environment, accelerate wound healing, reduce pain, and has an antimicrobial effect.

Our preliminary research investigated the safety and efficacy of biocellulose wound dressings containing silk sericin and PHMB, *in vitro* and *in vivo* [24]. The results showed that the optimal combination of silk sericin and PHMB could promote collagen type I synthesis and have antimicrobial activity. The efficacy of the dressing was compared with Bactigras® (0.5% chlorhexidine acetate in white soft paraffin) on full-thickness skin wounds in rats. The wounds treated with the biocellulose dressing showed a significantly greater wound size reduction than those dressed with Bactigras®. No inflammation or irritation was shown in rats [24].

Long-term wound healing of donor sites is one cause of chronic wounds and patients may suffer from restricted motion that affects their daily activities. Therefore, the purpose of this study was to investigate the safety of a biocellulose wound dressing containing silk sericin and PHMB in healthy volunteers and the

clinical efficacy of this dressing for STSG donor site wound treatment compared with Bactigras®, the standard dressing for this type of wound at King Chulalongkorn Memorial Hospital, by monitoring the time required for complete re-epithelialization, wound quality, rate of infection, pain, and adverse events.

1.2 Research questions

Phase I clinical study

1. Does the biocellulose wound dressing containing silk sericin and PHMB irritate or inflame skin more than Bactigras® in healthy volunteers?
2. Does the biocellulose wound dressing containing silk sericin and PHMB induce more adverse events than Bactigras® in healthy volunteers?

Phase II clinical study

1. Does the biocellulose wound dressing containing silk sericin and PHMB show better efficacy than Bactigras® for STSG donor site treatment?
2. Can the biocellulose wound dressing containing silk sericin and PHMB reduce the pain levels compared to Bactigras® for STSG donor site treatment?
3. Are there any adverse events using the biocellulose wound dressing containing silk sericin and PHMB for the treatment of STSG donor sites?

1.3 Objectives

Phase I clinical study

1. To investigate the irritation or inflammation of healthy volunteers' skin covered with the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras®.
2. To investigate the adverse events occurring in healthy volunteers' skin covered with the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras®.

Phase II clinical study

1. To investigate the wound healing time of STSG donor sites treated with the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras®.

2. To investigate the wound quality of STSG donor sites treated with the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras®.

3. To evaluate the number of infections in STSG donor sites treated with the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras®.

4. To assess the pain levels of STSG donor sites treated with the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras®.

5. To indicate the adverse events occurring from the biocellulose wound dressing containing silk sericin and PHMB treatment for STSG donor sites and Bactigras®.

1.4 Hypotheses

Phase I clinical study

1. The irritation or inflammation of healthy volunteers' skin covered with the biocellulose wound dressing containing silk sericin and PHMB is less than or equal to Bactigras®.

2. The adverse events occurring in healthy volunteers' skin covered with the biocellulose wound dressing containing silk sericin and PHMB are less than or equal to Bactigras®.

Phase II clinical study

1. The wound healing time of STSG donor sites treated with the biocellulose wound dressing containing silk sericin and PHMB is less than or equal to Bactigras®.

2. The wound quality of STSG donor sites treated with the biocellulose wound dressing containing silk sericin and PHMB is higher than or equal to Bactigras®.

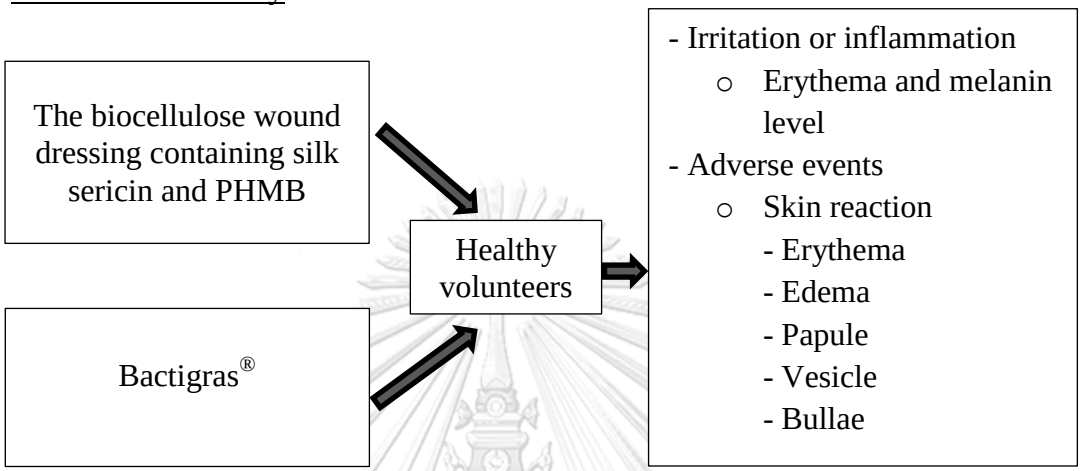
3. The number of infection in STSG donor sites treated with the biocellulose wound dressing containing silk sericin and PHMB is not more than Bactigras®.

4. The pain levels of STSG donor sites treated with the biocellulose wound dressing containing silk sericin and PHMB is less than or equal to Bactigras®.

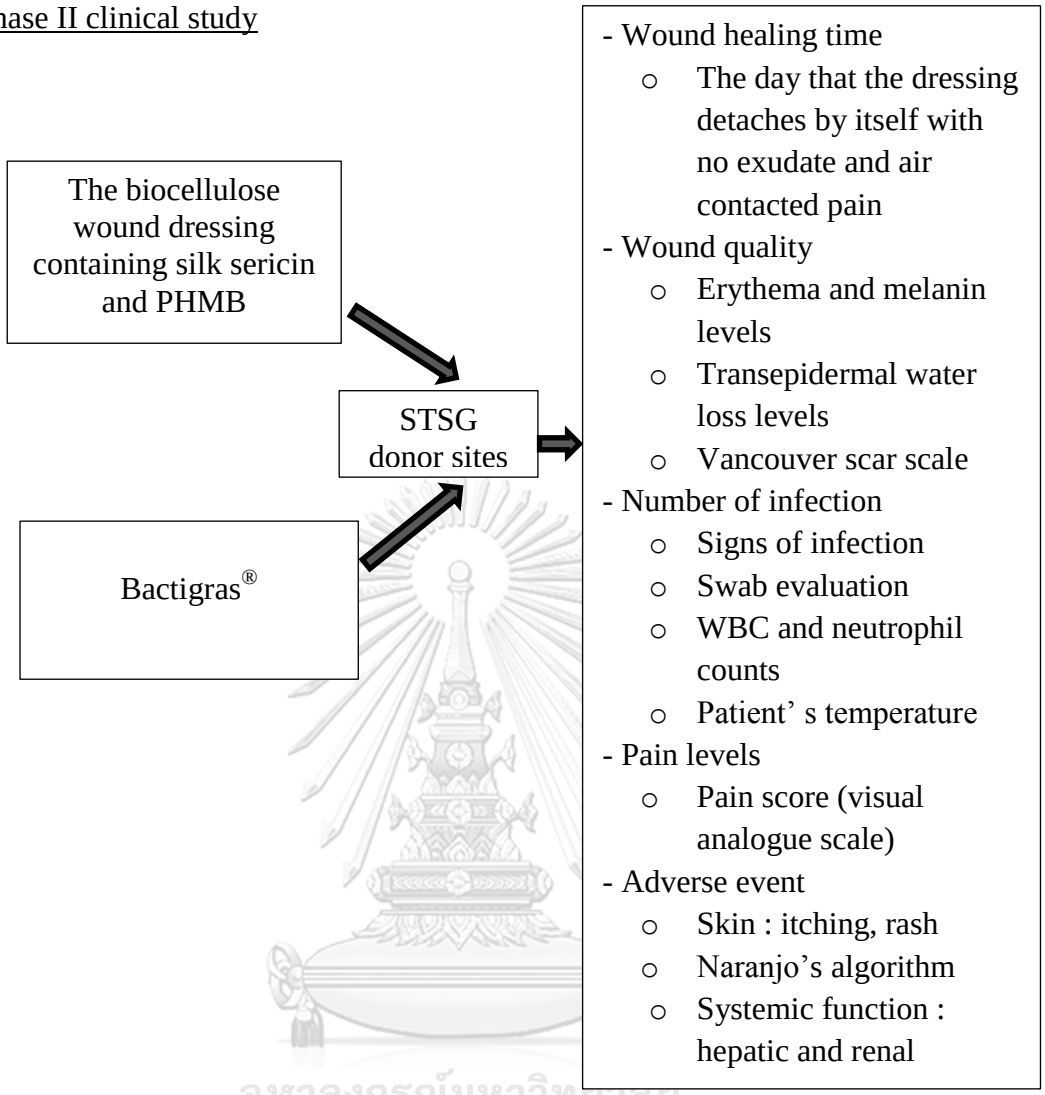
5. There are no significant adverse events occurring from the biocellulose wound dressing containing silk sericin and PHMB treatment for STSG donor sites and Bactigras®.

1.5 Conceptual framework

Phase I clinical study



Phase II clinical study



CHAPTER 2 LITERATURE REVIEW

2.1 Skin grafts donor site

Skin grafting is a process used for wound healing treatment since more than 3000 years ago [25]. It is a common procedure for skin reconstruction and usually the first contemplation when primary wound care cannot be practiced or there is insufficient surrounding skin to cover the wound [26]. Grafts are pieces of skin that have been completely detached from their local source and transferred to a wound at another site. The grafts survive on the wound where new vascular connections are created and nutrients are diffused to them [27]. Skin grafts are divided into two types according to their thickness, split-thickness and full-thickness [28]. The sites from which skin is removed are called donor sites. These sites should be treated with proper dressings for wound healing acceleration and infection protection.

1. STSG donor sites

An STSG affects the epidermis and some part of the dermis. Its thickness is around 0.15–0.6 mm [1]. Therefore, the depth of an STSG donor site wound is the same as the graft thickness. Moreover, this type of donor site is closely related to a second-degree burn, so dressings are used as the major treatment device. If appropriate care is received, the wound will recover in 7–14 days [2]. However, there are many factors that have an effect on healing including age, comorbidity, and complications. A donor site wound treated unsuitably may lead to a deeper or infected wound that may result in systemic treatment requirement. Any STSG with a slow healing rate may cause hypertrophic scarring, so wound healing acceleration is most beneficial for the wound healing process [29]. Accordingly, this research will study STSG donor site wounds.

2. FTSG donor sites

An FTSG includes the epidermis and dermis but does not cover subcutaneous fat which is a barrier to nutrient supply. Its thickness is more than 0.6 mm [1]. Most donor site wounds are treated with a suture procedure, so there are fewer requirements for dressings.

2.1.1 STSG donor site assessment

STSG donor site was assessed in term of wound healing efficacy, pain score, number of infections and adverse events.

1. Wound healing efficacy

Wound healing efficacy was evaluated in four conditions. First, wound healing was percentage of wound re-epithelialization or closure of more than 95% [30, 31] or 100% [32, 33] by at least two experienced wound specialists assessment [33]. Second, wound healing referred to wound healing time which was the day that the dressing detached by itself with no exudate and no air-contacted pain [13, 34]. Third, wound healing referred to number of wound requiring debridement, skin graft, and secondary excision [35]. The last condition, wound healing was evaluated in term of time of dressing change [36]

2. Pain score

Pain score was assessed using Visual Analogue Scale (VAS) [33, 37, 38] or Johns Hopkins Pain Rating Instrument [31] at 10–15 min before [20, 21, 30, 33, 35], during [20, 30], or 10–15 min after dressing change [20, 21, 33]. Moreover, pain also evaluated in term of frequency of pain medication requirement or number of pain medication doses per day [38].

3. Number of wound infections

Number of wound infections was evaluated in terms of signs of infection (erythema, swelling, malodor, tenderness, cellulitis, or visible purulent discharge [30, 31, 35, 38]), wound swab test [31, 38], WBC and neutrophil counts, and patient's temperature. There were many techniques for wound swab test including Levine technique [21] and sterile cotton-tipped swabs drawn over the wound 10 times in a zigzag pattern [30]. In the previous research, swab test were test every 6 days [32], every week [30, 39] or twice a week [35]. WBC and neutrophil counts were evaluated. The WBC and neutrophil count that were higher than normal range were recorded to be infection wound [40]. Moreover, patient's temperature (more than body temperature) was one of infection signs [41].

4. Adverse reactions

Adverse reactions were assessed in terms of allergic reactions (five-point scale was clearly relevant, probably relevant, probably irrelevant, surely irrelevant, and unable to determine.) [32], maceration and redness (four-point scale was none, slight, moderate, and high.) [21], general tolerability score (four-point scale was very good, good, moderate, and poor.) [21], irritation and maceration at periwound skin (0–10 scale) every dressing change [30], and systemic reactions (blood, urine, liver, and kidney function tests) [32].

2.1.2 STSG donor site wound management

Donor sites can be practically anywhere in the body. However, donor sites are usually selected in an area under clothing and with similar characteristics to the recipient site. The thigh is the most common area for STSG donor sites [27] among other areas including the scalp, buttock, back, upper arm, forearm and abdominal wall [28]. There are many factors which affect STSG donor site wound healing including the depth, site, and size of the wound, along with the age of the patient and comorbidity [4]. Therefore, appropriate wound management before and after surgery is required.

Preparation of the donor site before surgery [27]:

1. Clean the area with antibacterial solution.
2. Wash off the antibacterial solution with saline and dry the area.
3. Apply a sterile lubricant to the area such as mineral oil or K-Y jelly.

The goals of STSG donor site wound treatment are [3, 4]:

1. Accelerated wound healing
2. Infection prevention
3. Pain reduction
4. An optimal environment to promote healing

The donor site heals by a process of re-epithelialization. The healing process is composed of three major phases which are inflammation phase, proliferation phase, and regeneration phase. The inflammation phase is around 1–3 days. Wound exudate control and wound sterilization should be carried out. After 4–14 days of injury, the proliferation phase will occur. Fibroblasts migrate

into the wound and collagen is synthesized. Moist wound management with dressings and pain control are important. The last phase is the regeneration phase. It occurs around 14–21 days after injury. Tissue differentiation, wound contraction, and scar formation are the main processes. Importantly, moist wound management will promote wound healing and good scar quality. Topical antimicrobial agents or dressings used as primary dressings are applied onto the wound, based on the exudate level and infection risk after operation. The ideal method of STSG donor site wound treatment is one dressing, which should be applied to the donor site and left in contact until the wound is healed, to avoid damage to the new epithelium [28]. Pain management with adequate pain medication is regularly used. Signs of infection – pain, redness, warmth, swelling, and odor – should be evaluated every day. A wound swab test for microbial culture such as the Levine technique is the other indicator to support infection assessment [42]. Biopsy of tissue from the wound is used to indicate a specific microorganism. Blood culture is used for systemic infections. Blood pressure, heart rate and urine output should be observed. Baseline laboratory results including hematocrit, urinalysis, electrolytes, and chest x-ray should also be followed up. During treatment, patients may have complications such as renal failure, gastrointestinal bleeding, and sepsis so they should be closely monitored for these effects. Long-term treatment should prevent wound contraction and hypertrophic scarring. After healing, emollient agents may reduce itching. Sun block cream is useful for burning protection.

2.2 Topical STSG donor site dressings

Many dressings are available for the treatment of STSG donor site wounds. Moist wound dressings seem to have more advantages than other dressings [3]. Traditional dressings or non-moist wound dressings include mesh gauze and petrolatum gauze. These dressings allow airflow and allow the exudate to dry. They may adhere to the wound surface, lead to pain, and delay wound healing [4]. For high exudate, wounds require covering with secondary absorbent dressings. However, excessive absorbance also leads to dry wounds and delayed wound healing.

Ideal STSG donor site dressing properties are [43]:

1. Outer environment and infection protection

A dressing is a barrier to protect the wound from the external environment. A bad environment delays wound healing and may cause a chronic wound. Microorganisms are the major cause of inflammation and failure of the wound healing process. Moreover, uncontrolled mechanical strength from outside is the other cause of wound healing disturbance including cell proliferation, migration, and differentiation. Therefore, the protection property of a dressing preserves a suitable setting for the wound healing process.

2. Non-adherent and safe

In the wound healing process, new cells such as fibroblasts and keratinocytes are generated to close the wound. More than outer environment protection, the dressing should preserve the appropriate inner environment, cells and substances for wound healing. However, an adherent dressing eliminates important cells and factors, leading to non-healing and inflammation. Consequently, a non-adherent dressing will protect the suitable inner environment of the wound. Moreover, safety is the most concerning factor in all materials that are in contact with humans. A dressing that is highly efficient but highly toxic is unacceptable.

3. Gas exchange

Insufficient oxygen is a cause of delayed wound healing. Oxygen is an important factor in energy production. This production preserves sources of energy for cell function together with proliferation, migration, differentiation, and angiogenesis [44]. A dressing should allow gas exchange support and balance oxygen and other gases for the wound healing process.

4. Keeping the wound moist

Moist wound dressings have many advantages. They can prevent the drying and deepening of wounds. They minimize risks from mechanical damage. Moreover, they can promote an optimal environment, resulting in rapid wound healing [3]. Therefore, moist wound dressings are suitable for STSG

donor site wound treatment. Voineskos *et al* carried out a systematic review of skin graft donor site dressings, comparing moist wound dressings with non-moist wound dressings [45]. The majority of studies showed that moist wound dressings had a faster healing rate, less pain, and were less expensive. However, the infection rate was not significantly different. These results agreed with the study of Wiechula about the use of moist wound-healing dressings in the management of STSG donor sites [3]. The number of days taken for wound healing with hydrocolloid dressings was significantly lower than for other moist products. However, pain and infection rate were not significantly different.

5. Non-inhibition of the wound healing process

The ideal dressing should not disturb the wound healing process. Disruption may delay wound healing, leading to a chronic wound.

6. Painless application

Pain is the one of concerning factors because it has an effect on patients' quality of life. It may influence patient compliance or acceptance. Painless application provides a benefit and is a good choice for wound treatment.

7. Ease of application and comfort

These properties are useful for patients and caregivers to improve compliance. They may help to decrease the time taken to change the dressing and decrease the opportunity for wound contamination. Good dressing design is important to ensure these properties.

8. Cost-effectiveness

Cost is the major problem for dressing selection. Because of financial problems, most patients in Thailand cannot be treated with costly dressings despite their having higher benefit than low-cost dressings. A costly dressing may not be highly successful in the market.

Many types of commercial dressing including composition, advantages, and disadvantages are shown in Table 1.

Table 1 Types of dressings, advantage and disadvantage of the dressings [46-56]

Type of dressings	Commercial products	Dressing Compositions	Wound type treatments	Advantages								
				Environment protection	Non-adherent	Control gas and water exchange	Moist wound keeping	Wound healing acceleration	Painless	Wound exudate absorption	Wound debridement	Others
1. Biological dressings	Trancyte™ Integra® Apligraf® Alloderm®	Biologic products from human or animal	Full or deep wound	+		+		+	+			Closely with real skin
				Disadvantages -Short shelf life -No antibacterial activity -Appropriate for cleaned wound								
2. Biosynthetic dressings	Biobrane® Calcium alginate Glucan II®	Biological products, synthesis polymers and chemical	Thin or deep wound	+		+		+	+			Closely with real skin
				Disadvantages -No antibacterial activity -Appropriate for cleaned wound								

Type of dressings	Commercial products	Dressing Compositions	Wound type treatments	Advantages								
				Environment protection	Non-adherent	Control gas and water exchange	Moist wound keeping	Wound healing acceleration	Painless	Wound exudate absorption	Wound debridement	Others
3. Bacterial cellulose	XCell® Suprasorb® X	Bacterial cellulose is produced by *Acetobacter xylinum*.	-Low to moderate wound exudate -Partial thickness and full thickness wound	+	+	+	+	+	+	+	+	-Natural products -Ultrafine network structure -Good to be a medicine deliver
				Disadvantages -May loss of properties and difficult to rehydrate when it is dry -Should keep in humid state								
4. Synthetic dressings												

Type of dressings	Commercial products	Dressing Compositions	Wound type treatments	Advantages								
				Environment protection	Non-adherent	Control gas and water exchange	Moist wound keeping	Wound healing acceleration	Painless	Wound exudate absorption	Wound debridement	Others
4.1 Foam dressings	Allevyn™ Biatain®	Hydrophilic polyurethane or polyvinyl alcohol foam sheets	-High wound exudate -Partial thickness, full thickness, deep wound	+	+		+		+	+++		Extend time of wound dressing change (3-5 days)
				Disadvantages -The excess absorption leads to wound dehydration, adherence and infection -Not suitable for low or no exudate wound -May cause of peri-wound maceration								

Type of dressings	Commercial products	Dressing Compositions	Wound type treatments	Advantages								
				Environment protection	Non-adherent	Control gas and water exchange	Moist wound keeping	Wound healing acceleration	Painless	Wound exudate absorption	Wound debridement	Others
4.2 Hydrofiber dressings	Aquacel®	Sodium carboxymethyl cellulose	-High wound exudate -Partial thickness, full thickness, deep wound	+			+		+	+ ++		Extend time of wound dressing change (2-7 days)
				Disadvantages -The excess absorption leads to wound dehydration, adherence and infection -Not suitable for low or no exudate wound -Fiber may split out and stay into the wound. -Required secondary dressing								

Type of dressings	Commercial products	Dressing Compositions	Wound type treatments	Advantages								
				Environment protection	Non-adherent	Control gas and water exchange	Moist wound keeping	Wound healing acceleration	Painless	Wound exudate absorption	Wound debridement	Others
4.3 Alginate dressings	Algisite M Comfeel® Kaltostat®	Calcium alginate which is exacted from seaweed	-Moderate to high wound exudate -Partial thickness and full thickness-wound	+		+	+			++	+	Extend time of wound dressing change (2-7 days)
				Disadvantages -The excess absorption leads to wound dehydration, adherence and infection -Not suitable for low or no exudate wound -It may separate to small pieces and difficult to peel it out -Required secondary dressing and high cost								

Type of dressings	Commercial products	Dressing Compositions	Wound type treatments	Advantages								
				Environment protection	Non-adherent	Control gas and water exchange	Moist wound keeping	Wound healing acceleration	Painless	Wound exudate absorption	Wound debridement	Others
4.4 Hydrogels	Flexigel™ Duoderm® gel Intrasite gel	Organic polymers and water	-Low wound exudate -Partial thickness and full thickness-wound		+		+ +		+	++	+	Donate moist to the wound
				Disadvantages -Not suitable for high exudate -Required secondary dressing -Difficult to keep in place on superficial wound								

Type of dressings	Commercial products	Dressing Compositions	Wound type treatments	Advantages								
				Environment protection	Non-adherent	Control gas and water exchange	Moist wound keeping	Wound healing acceleration	Painless	Wound exudate absorption	Wound debridement	Others
4.5 Hydrocolloid dressings	Duoderm® Urgotul® Tegasorb™	Sodium carboxymethyl cellulose (CMC), pectin, and gelatin	-Low to moderate wound exudate -Partial thickness and full thickness-wound	+	+		+		+	++	+	Extend time of wound dressing change (2-7 days). - Water proof ability
				Disadvantages -The dressing should be changed within day in moderate wound exudate treatment. -Sticky to caregiver glove								

Type of dressings	Commercial products	Dressing Compositions	Wound type treatments	Advantages								
				Environment protection	Non-adherent	Control gas and water exchange	Moist wound keeping	Wound healing acceleration	Painless	Wound exudate absorption	Wound debridement	Others
4.6 Film dressings	Tegaderm™ Opsite®	Transparent adhesive films	-Very low wound exudate -Superficial wound	+		+	+				+	-Water resistance ability -Permit evaluation of wound progress without removal of dressing
				Disadvantages -Not suitable for moderate to high exudate -May tear healthy skin -Non absorptive dressing								

2.3 Biocellulose

2.3.1 Physical and chemical properties of biocellulose

Biocellulose is a type of cellulose produced by *Acetobacter xylinum*, a Gram-negative acetic acid bacterium [57]. It uses oxygen and carbon sources including glucose for biocellulose production in static optimal medium with pH 4–6 [58-60]. Dahman *et al* found that fructose medium produced the highest biocellulose production, close to that of a sugar mixture medium of glucose, xylose, arabinose, galactose, and mannose [61]. Static biocellulose culture conditions promote more extension fibrils than agitated conditions. Fibrils of biocellulose in static culture accumulate on the medium surface because of high oxygen levels [58]. Biocellulose has the chemical structure $(C_6H_{10}O_5)_n$, like plant cellulose [62]. It has an ultrafine network structure of β-1,4 glucan chain bundle microfibrils of around 3–8 nm [63]. Biocellulose exists in Iα and Iβ crystalline forms, with the Iβ form mainly found in plant cellulose [6]. The properties of biocellulose are higher purity, crystallinity (more than 60%), degree of polymerization, and tensile strength than plant cellulose [6, 64, 65]. Because of the nanofiber structure of biocellulose, it can hold a large amount of water, more than 200 times its dry weight, while being high elastic [5]. In addition, biocellulose can contain medication and release it to the target area because of its many nanopores [62, 66]. Therefore, biocellulose is used in many applications including tissue engineering, headphone diaphragms, paper, and wound dressing [67]. However, biocellulose may lose properties and be difficult to rehydrate when it is dry, so it should be kept in a humid state [6]. The advantages and disadvantages of biocellulose compared with other types of dressing are shown in Table 1.

2.3.2 Safety and efficacy of biocellulose

Biocellulose has many advantages for wound healing which are transparency, autolytic debridement, acceleration of re-epithelialization, and fewer daily wound dressing changes [5]. Because of the water-holding capacity of biocellulose, it can retain moisture, which is a good environment for wound healing. It can be a barrier to protect the wound, deliver medicine to

the wound, and is biocompatible [67]. Moreover, the ultrafine networks of biocellulose are dense so they are barriers for cell migration into the material, which leads to pain reduction [6].

In an *in vivo* study on the biocompatibility of biocellulose, biocellulose was implanted subcutaneously in 21 Wistar rats for 1, 4, and 12 weeks. The results of the study showed no signs of inflammation, foreign body reaction, abnormal cell ingrowth, or abnormal angiogenesis in the biocellulose implantation area. Therefore, it is safe and can be used as a dressing material [68]. Mendes *et al*. also studied the tissue reaction of subcutaneous biocellulose implantation in mice at 15, 30, 60, and 90 days. They found a mild inflammatory response and few lymphocytes until 30 days of implantation. However, foreign body reaction and penetration of connective tissue into the membrane were not observed at any time [69]. Park *et al*. studied the safety and efficacy of biocellulose in rats. For the safety test, six rats were implanted with biocellulose in the back and the other six rats were not implanted but had the same incision. They found that no signs of inflammation, laboratory abnormality, or liver or kidney toxicity were observed in the biocellulose group after 28 days. For the efficacy test in 20 rats at 7, 14, and 21 days, each rat was incised with three full-thickness wounds on their back and the wounds were treated with dressings of Vaseline gauze (control group), Algisite M® or biocellulose. After 15 days, the wound area in the biocellulose group was significantly less than the Vaseline gauze and Algisite M® groups (7.2% vs 33.0% and 14.5%). Neutrophil levels were significantly lower in the biocellulose group than the control group. Therefore, they concluded that biocellulose contributed to wound healing and was not toxic, and could therefore be used as a dressing material [70].

Biocellulose also showed several advantages as a biological dressing and had been suggested for use as a temporary skin substitute in wound treatment [71]. Moreover, biocellulose was suitable for use as a medicine-delivering dressing. Clinical studies about biocellulose for wound healing are shown in Table 2.

Table 2 Clinical studies of biocellulose for wound healing

Study design	Wound type	Number of subjects	Study treatment	Control treatment	Outcomes	Side effects
Randomized controlled trial [9]	Chronic venous insufficiency and lower leg ulceration	24 (Sample 12, control 12)	Bacterial cellulose dressing plus a two-layer compression bandage	A non-adherent petrolatum emulsion impregnated cellulose acetate gauze plus a two-layer compression bandage	- Healing time (>75% re-epithelization of the wound treated with bacterial cellulose group was lower than that of the control group (43 VS 71 days, respectively). - Percentage of wound size reduction of the wound treated with bacterial cellulose group was higher than that of the control group after 6 and 12 weeks (39% VS 19%, 74 VS 49%, respectively). - Pain score of the wound treated with bacterial cellulose was significant lower than that of the control group after 3, 6, and 8 weeks. - Autolytic debridement of bacterial cellulose treated group was significant higher than control group.	Leg edema, wound exudates, wound odor, and maceration are not different in both groups.
Randomized controlled trial [25]	Critical colonized or locally infected wounds	38 (Sample 21, control 17)	Bacterial cellulose dressing containing PHMB	Silver dressing	- Pain score of the wound treated with bacterial cellulose was significant lower than that of the control group after day 1, 3, 7, 14, 21, and 28. - Score of quality of life of the wound treated with bacterial cellulose was higher than control group. - Bacterial reduction of the wound treated with bacterial cellulose was significantly higher and faster than control group.	No adverse reactions were presented in both groups

Study design	Wound type	Number of subjects	Study treatment	Control treatment	Outcomes	Side effects
Randomized controlled trial [37]	Partial thickness burn patients	60 (Sample 30, control 30)	Bacterial cellulose dressing containing PHMB	Silver sulfadiazine cream	- Pain score of the wound treated with bacterial cellulose was significant lower than that of the control group. - There was no significant difference of healing time between the bacterial cellulose dressing and silver cream group. - The bacterial cellulose dressing could save cost of treatment better than silver sulfadiazine cream	No report
Uncontrolled before and after study [24]	Non-healing biofilm wound (no response to standard treatment for 2 weeks)	16	Bacterial cellulose dressing containing PHMB	-	- Complete epithelialization with no drainage was found 12 wounds (75%) after 24 weeks. - Slough in the wound bed at week 24 was less than day 0. (23% VS 62%). - The mean percentage of granulation tissue at week 24 was significantly higher than day 0 (77% VS 38%). - Pain score of all patients decreased after dressing change.	No report
Case report [10]	Face partial thickness burn wound	1	Bacterial cellulose dressing	-	- The wound that was treated with bacterial cellulose dressing only once healed with full epithelialization after 2 weeks. - Bacterial infection did not found.	No allergic reaction or irritation

2.4 Wound healing accelerators derived from animals

Wound healing accelerators derived from animals, including chitosan, collagen, honey, anabolic steroids, silk sericin, peptides, and proteoglycan were reviewed and compared in terms of their mechanisms of action, advantages, and disadvantages when applied in topical applications [72].

2.4.1 Chitosan

Chitosan is a deacetylated derivative of chitin which is mostly found in the exoskeletons of arthropods including shrimp, crab, and insects [73]. It exists in alpha, beta, and gamma forms; the different characteristics of the three forms are summarized in Table 3. Chitosan is a polysaccharide composed of β-(1→4)-linked glucosamine and N-acetyl-D-glucosamine. It has a molecular weight between 50 and 2000 kDa. The physical and chemical properties of chitosan depend on the degree of acetylation and acetyl group distribution [74]. Chitosan is soluble in acidic solution and insoluble in high pH environments because the amine group is deprotonated and loses its charge [75]. Chitosan is biodegradable; that with a low degree of deacetylation or low molecular weight is degraded more rapidly [75]. Chitosan is also biocompatible and has low toxicity. It was reported that the glucosamine units of chitosan are an effective wound healing accelerator [76]. Therefore, various forms of chitosan are widely used for wound healing including powders, gels, films, fibers, and scaffolds. The commercial products of chitosan and its derivatives for wound healing are Tegasorb®, Tegaderm®, HemCon Bandage™, Chitodin®, and Trauma Dex® [77].

2.4.1.1 Mechanisms of wound healing

Chitosan promotes wound healing through two major pathways. Firstly, the N-acetyl-D-glucosamine unit of chitosan initiates fibroblast proliferation and collagen production [76]. The positive charge of chitosan has an electrostatic interaction with glycosaminoglycans, leading to growth factor attraction [78]. Secondly, macrophages are activated by N-acetyl-D-glucosamine, leading to phagocytosis and the release of mediators including TGF-β1

and platelet-derived growth factor (PDGF). These biological mediators subsequently accelerate extracellular matrix (ECM) synthesis [79]. Moreover, chitosan activates the production of IL-1 which controls fibroblast proliferation and collagen synthesis [80]. It is also reported that chitosan stimulates the release of IL-8 by fibroblasts, which leads to angiogenesis and migration of neutrophils. Therefore, it helps in faster wound healing and scar prevention [77]. Previous studies on chitosan are shown in Table 4.

2.4.1.2 Antibacterial effect

The chitosan structure is composed of amine groups (cationic polymers) which can strongly bind to anionic proteins in the cytoplasmic membrane of bacterial cells, leading to an imbalance of the cell membrane and cell death [77]. Moreover, low molecular weight chitosan (less than 5 kDa) can penetrate the bacterial cell wall, combine with DNA, and inhibit mRNA and DNA transcription [81]. Differences in the antibacterial mechanism of chitosan against Gram-positive and Gram-negative bacteria have been reported. Zheng *et al* found that the antimicrobial activity of chitosan against Gram-positive bacteria (*S. aureus*) increases when the molecular weight is increased because high molecular weight chitosan can form an external barrier to block nutritional intake. Nevertheless, the antimicrobial activity of chitosan against Gram-negative bacteria (*Escherichia coli*) increases when the molecular weight is decreased because low molecular weight chitosan easily enters bacterial cells [82]. Chitosan with a low pH (pH < 6.5) and a low degree of acetylation is also more rapidly absorbed into the bacterial cell wall and chitosan cations are augmented [74], [83].

2.4.1.3 Biological safety

The degree of deacetylation of chitosan has no significant influence on keratinocyte and fibroblast cytocompatibility [84]. Chitosan dressing materials including gels, membranes, and sponges were proved to be safe *in vivo* and showed no erythema, irritation, or

toxicity [85-87]. The chitosan sponge-treated group had no irritation on rabbit skin, which was comparable to the Vaseline gauze-treated group [85].

2.4.2 Collagen

Collagen protein is an important component in the human ECM of skin, bone, and other tissues. It can also be extracted from many kinds of animal including cows, pigs, and marine animals (fish, jellyfish, starfish, and squid). Table 5 shows the types, properties, and limitations of collagen obtained from different sources. The basic structure of collagen is composed of three polypeptide chains formed into a triple helix, and it has a molecular weight around 300 kDa [88]. The amino acid sequence of collagen contains an arginine-glycine-aspartic acid motif, a specific cell adhesion domain which promotes cell growth, differentiation, and activity [89]. Collagen is biocompatible and safe. However, it has a fast rate of biodegradation and low denaturation temperature. Some denatured collagen such as gelatin may lose its wound healing properties in production [90].

2.4.2.1 Mechanisms of wound healing

Collagen plays a chemotactic role to attract skin fibroblasts to the wound [91, 92] and supports fibroblast activity to produce granulation tissue. It can cross-link with fibronectin to generate an appropriate surrounding environment for the re-epithelialization process [93]. Moreover, collagen also binds platelets and generates thrombin in hemostasis [94], which is one of the important factors in inducing angiogenesis for the wound healing process [95]. Collagen types I and III are reported as the important factors in the wound healing process.

Normally, native collagen may be destroyed by elevated levels of matrix metalloproteinases (MMPs), leading to non-healing wounds [96]. Therefore, collagen dressings show a superior property by delivering substitute collagen to the wound and reducing the elastase level in the wound environment [97]. The collagen supports

chemotaxis action between fibroblasts and native collagen to promote natural wound healing [90]. Moreover, collagen dressings can absorb wound exudate and provide a moist wound environment [96]. Many collagen studies are shown in Table 6.

2.4.2.2 Biological safety

In terms of safety, adverse reactions of collagen to wound tissue are rarely found [98]. A few cases of IgE-mediated reaction of bovine collagen leading to allergic reaction have been reported. Conjunctival edema presented after highly purified bovine collagen contact with the eye during ophthalmic surgery [99, 100]. A bovine-derived type I collagen dressing showed development of dermatitis in three out of 20 cases [101]. Collagen dressings may induce overgranulation [102]. In addition, topical collagen products are expensive, so they may not be the best choice of wound dressing.

2.4.3 Honey

Honey is produced from nectar that is stored and transferred by bees to the honeycomb. The enzymes secreted from the bee influence the physical and chemical characteristics of honey. The main constituents of honey are sugar and water. The major sugar components in honey are fructose (38.2%) and glucose (31.3%) [103]. The other components including enzymes, amino acids, organic acids, carotenoids, vitamins, minerals, and aromatic substances [104]. These components vary depending on botanical origin, geographic origin, storage conditions, and temperature [105]. The composition of honey also has an effect on its physical and chemical properties. For example, the water content in various types of honey leads to differentiated viscosity, crystallization, color, flavor, specific gravity, and solubility [104]. Other physical properties also darken the color. Honey has an acidic pH of around 3.2–4.5 [104]. It has a hygroscopic property to absorb water from the environment; because of its water content (18.8%), it can absorb moisture from the environment at humidity levels above 60% [106]. Various types of honey such as manuka honey, fynbos honey, and tualang honey, obtained from different trees or nectar, have been

studied for wound treatment. Examples of clinical studies of topical honey treatment are shown in Table 7.

2.4.3.1 Antibacterial effects

Honey is reported as a bacteriostatic and bactericidal agent, depending on its concentration. A low concentration of honey (4–8% v/v of honey and 5–11% v/v of manuka honey) showed bacteriostatic properties while a high concentration of honey (5–10% v/v of honey and 8–15% v/v of manuka honey) had bactericidal properties [106]. Honey contains a high concentration of sugar, so it has high osmolality. It draws fluid out of the environment to create an unsuitable environment for the existence of organisms [107]. The low pH of honey (pH 3.2–4.5) creates an inappropriate environment for the growth of microorganisms [106]. Another mechanism of honey for antibacterial activity is hydrogen peroxide production. Hydrogen peroxide is a broad-spectrum antimicrobial molecule that is produced by glucose oxidase enzyme from bees. The hydrogen peroxide produced from bees is not toxic to the cells due to its low concentration, which differs from chemically synthesized hydrogen peroxide [108]. However, the osmolality may not be sufficient when honey is diluted or interacts with the enzyme catalase in wound exudate [109]. Therefore, bee honey may not be suitable for the treatment of high-exudate wounds. On the other hand, manuka honey has a methylglyoxal component as an antimicrobial agent that is unrelated to hydrogen peroxide production [110]. It also shows an antibacterial effect against biofilm and various microorganisms such as MRSA, *S. aureus*, *P. aeruginosa*, and *E. coli* [111]. In addition, honey is reported to reduce wound odor and has debridement activity [107].

2.4.3.2 Anti-inflammation effects

An excessive inflammatory reaction can delay the wound healing process. Honey can reduce the extent of inflammation. Honey suppresses activity of protease, an important enzyme in the inflammatory process. The acidic property of honey produces a low pH

environment that is not appropriate for protease activity [112]. It increases nitric oxide which plays an important role in angiogenesis and the healing process. It also decreases prostaglandins in blood circulation and reduces inflammation and pain in the wound [113]. Moreover, honey contains antioxidants including flavonoids, phenolics, and vitamin C which interact with reactive oxygen species and reduce cellular damage [108, 114].

2.4.3.3 Biological safety

Honey is non-toxic, non-allergic, and non-irritating to wound tissue [115]. However, there are some cautions of use. Patients who have a history of pollen allergy may be allergic to honey. Furthermore, dehydration of tissue may be found in extensive honey treatment. Some patients may be sensitive to the acidity of honey [106]. Moreover, honey for medical treatment has to be sterilized by gamma irradiation to kill bacterial spores [107].

2.4.4 Anabolic steroids

Anabolic steroids are steroidal androgenic hormones, usually steroid hormones, obtained from either chemical synthesis or animal parts. Natural anabolic steroids are produced in pigs, boars, horses, and sheep [116]. Among these, pigs are the most common source of anabolic steroids for wound healing, especially porcine placenta and testis. Anabolic steroids are composed of four aromatic base structures, three cyclohexane rings and one cyclopentane ring. There are more than 100 possible substances created following molecular substitution to the ring base. For porcine testis extracts, the steroids with the highest content are 19-nortestosterone (nandrolone) (MW = 274.4 g/mol), testosterone (MW = 288.43 g/mol), and 17β-estradiol (MW = 272.39 g/mol) [117]. They are easily soluble in alcohol but hardly soluble in water. Moreover, they have low stability and are easily oxidized [118-120]. Topical anabolic steroids have been used for wound treatment in the form of solutions and creams.

2.4.4.1 Mechanisms of wound healing

The activity of anabolic steroids increases net protein preservation in the wound for the new tissue formation process. They have an anticortisol activity which can decrease the catabolic response of cortisol, and decrease protein degradation without altering its anti-inflammatory response [121]. They also have a direct effect on insulin-like growth factor 1 (IGF-1), transforming growth factor beta (TGF-β), and fibroblast growth factor (FGF), which play important roles in the activation of cell proliferation, cell migration, collagen synthesis, tissue formation, and angiogenesis [117, 122, 123], leading to wound closure. Examples of studies on topical anabolic steroid treatment are shown in Table 8.

2.4.4.2 Biological safety

The number of clinical studies on topical anabolic steroids extracted from animals is very limited because most topical steroids are modified by chemical synthesis. One study assessed the toxicity of cream + porcine testis-extracted steroid in wound healing by counting white blood cells. There was no significant difference in WBC count between the cream + porcine testis-extracted steroid-treated group and the cream without porcine testis-extracted steroid-treated group. No skin rash was found in either group [117]. However, a limitation of using topical anabolic steroids is that the anabolic steroids can enter the blood circulation and cause systemic effects [124].

2.4.5 Peptides

Peptides are biological molecules that are important factors in enzymes, the healing process, and other metabolic functions of living organisms. They are polymers composed of amino acid residues linked with amide bonds (carboxyl group and α-amino group linking) [125]. The molecular weight of peptides is less than 6000 Da, less than that of proteins [125]. Peptides have low stability and a short half-life, so they are easily degraded. Biotechnology to adjust factors such as pH or temperature can control peptide stability [126]. Peptides can be

obtained by chemical synthesis or extracted from natural sources, especially amphibians. Amphibians such as salamanders and frogs have a special wound repair process. They can repair wounds by regeneration, like mammalian fetal skin, and show perfect wound healing. In contrast, the wound repair process in mammalian adult skin is scar formation, which leads to imperfect wound healing. Therefore, amphibian peptides have been extracted and applied for wound treatment. Tylotoin extracted from the salamander (*Tylototriton verrucosus*) is composed of 12 amino acid residues including two cysteines forming an intramolecular disulfide bridge [127]. AH90 is a peptide extracted from frog skin (*Odorrana grahami*). It is composed of 24 amino acid residues [128].

2.4.5.1 Mechanisms of wound healing

Peptides provide a function in wound healing. They bind cell-surface integrin, leading to cell adhesion and migration. They also trigger the invasion of fibroblasts or keratinocytes [129]. The transcription factor Prx1 is one of the key factors that activates fibroblasts in amphibian skin wound healing [130]. However, there are many peptides involved in this process. Previous research has investigated peptides extracted from salamander and frog skin as wound healing accelerators. The results showed that topical treatment with tylotoin extracted from salamanders increases proliferation and migration of keratinocytes, fibroblasts, and vascular endothelial cells. It also enhances secretion of TGF-β1 and IL-6, important factors in the wound healing process [127]. Liu *et al* found that AH90 extracted from frog skin at a concentration of 250 μg/mL significantly accelerates wound healing in mice compared with vehicle. It promotes re-epithelialization and TGF-β1 secretion to the wound, and increases cell adhesion to fibronectin and laminin [128]. *In vivo* tests are shown in Table 9.

2.4.5.2 Biological safety

The safety evaluation of peptides has not been reported. Further research on the sequence, stability, and physical properties of these peptides is required.

2.4.6 Proteoglycans

Proteoglycans (PGs) are complex extracellular macromolecules consisting of a core protein with one or more covalently attached glycosaminoglycans (GAGs) [131]. The chemical structure of PGs is complex. They are composed of many types of protein such as aggrecan, versican, and GAGs including hyaluronan, chondroitin sulfate, and keratin sulfate [131]. The properties of PGs also depend on their chemical structure. For example, aggrecan, with hyaluronic acid, is a structural component of ECM in cartilage, brain, intervertebral discs, tendons, and corneas [132, 133]. They provide water retention, osmotic pressure, and proper collagen organization to the cell. Small leucine-rich PGs are the most prevalent PGs found in ECM. They regulate cell processes including migration, proliferation, and angiogenesis. Decorin, a class I small leucine-rich PG, has an effect on collagen fibrillogenesis, fibroblast function, and wound healing [132, 133]. A part of cartilage, PG structures are similar to EGF-like module, a factor in the wound healing process [132, 133]. Among various sources of PGs, those extracted from salmon nasal cartilage are widely studied as wound healing agents [134]. Aggrecan with chondroitin sulfate is the major component of PGs extracted from salmon nasal cartilage [135]. Three globular domains and two GAG-attachment domains are provided in its core protein. It is a hydrated and viscous gel because of the attached chondroitin sulfate chains within its domain [136]. Chondroitin 6-sulfate, which has an effect on wound healing, is also found in PGs extracted from salmon nasal cartilage at around 60% compared with 40% in bovine PGs [137]. Therefore, bovine PGs are studied more for joint and other treatments than wound treatment [138, 139].

2.4.6.1 Mechanisms of wound healing

PGs bind growth factors, cytokines, enzymes, and ECM components including FGF, IL-8, EGF, and collagen [140], and regulate cell proliferation, migration, and angiogenesis in the tissue repair process and host defense mechanisms [141, 142]. In an *in vitro* test, PGs extracted from salmon nasal cartilage at concentrations of 0.1–10 µg/mL could stimulate fibroblast proliferation and migration, leading to wound healing

acceleration. The important mechanism for wound closure was the interaction between chondroitin sulfate in the PGs and the cell surface. This interaction activated the intracellular signaling pathway to promote cell proliferation and migration. Moreover, PGs extracted from salmon nasal cartilage contained core polypeptides such as EGF ligand to stimulate cellular proliferation and migration. However, this effect was reduced when the PG concentration was 10–1,000 µg/mL [134]. Cream containing 1% and 2% PGs extracted from fish cartilage was used to treat second-degree burn wounds in rats. The results showed that the percentage of wound healing in the PG cream-treated group was higher than the cream without PG group after 7, 14, 21, and 28 days. The amount of PG had an effect on wound healing. The cream containing a higher PG content (2%) promoted a higher percentage of wound healing than that with a lower content (1%). Re-epithelialization and neovascularization of the PG cream-treated group were found to be greater than the control. The number of giant cells and macrophages was not significantly different, and continuously decreased after 14 days [143].

2.4.6.2 Biological safety

There is no clinical report on the biological safety of using PGs for wound treatment. However, there was no abnormality in rats treated with salmon cartilage powder containing PGs (1,000 mg/kg/day) for 90 days. Moreover, there were no clinical side effects or abnormalities in blood tests in adults who received salmon cartilage powder containing PGs (1,500 mg/kg/day) for 5 days [144].

2.4.7 Sericin

Sericin is a protein extracted from silk cocoons of *Bombyx mori*. It contains 18 amino acids. Serine, a moisturizing acid, accounts for around 30% of the total amino acid content [14, 145, 146]. Following serine, aspartic acid and glycine are also found, at around 10–20% [146, 147]. The molecular weight of silk sericin depends on the extraction method. Silk sericin obtained from heat extraction using an autoclave at 120 °C for 60 min has a molecular weight

around 25–150 kDa [147]. Silk sericin is a hydrophilic molecule which is water-soluble. [148]. The secondary structure of silk sericin is composed of two forms, a random coil and a β-sheet. At high temperatures (more than 50 °C), the major form of silk sericin is a random coil which is easily soluble. On the other hand, at lower temperatures, the major form of silk sericin is a β-sheet which has strong hydrogen bonds, making it more difficult to dissolve and which may form a gel upon cooling [14]. Recently, various biological properties of silk sericin have been elucidated such as wound healing, antibacterial effect, and anti-inflammatory properties. It was then introduced for use in medical and pharmaceutical applications in various forms such as lotions, creams, ointments, gels, dressings, and bandages [14]. Sericin has been widely studied as an acceleration agent for wound healing. Examples of clinical studies on topical wound treatment using silk sericin are shown in Table 10.

2.4.7.1 Mechanisms of wound healing

Silk sericin is reported to accelerate proliferation of fibroblast cells and collagen synthesis [9]. Silk sericin from heat extraction using an autoclave at 120 °C for 60 min activated the highest production of type I collagen and showed the lowest toxicity to cells at a concentration range of 8–100 µg/mL. Silk sericin from Chul Thai Silk 1/1 (0.2–1.0 mg/mL) can activate growth of L929 mouse fibroblast cells and the production of collagen type I in cell culture medium [10]. It could increase the number of skin fibroblast cells and collagen production to 250% of non-sericin levels after 72 h [149]. Furthermore, it accelerated proliferation of mammalian cells including human epithelial and human hepatoblastoma cells [146]. Moreover, it can increase skin elasticity, and has an anti-aging effect.

2.4.7.2 Antibacterial effects

Some research found that silk sericin can inhibit bacterial growth. Fabric coated with silk sericin exacted by ethanol could inhibit *S. aureus* and *E. coli* in a disk diffusion test. Moreover, it decreased these bacteria by more than 80% in a broth test [150]. Silk sericin extracted by an alkali process (0.25% sodium bicarbonate) showed

potent antibacterial activity to *E. coli*. Silk sericin extracted by a water degumming process possessed antibacterial activity to *S. aureus*. It caused membrane dysfunction and disturbed division and growth of both *E. coli* and *S. aureus* [151]. It also decreased colonies of Gram-positive bacteria when the sericin concentration was increased [152]. However, some research showed that purified silk sericin extracted by a degumming process without Tris-HCl and alkali does not have an antibacterial effect [153].

2.4.7.3 Anti-inflammation effect

Silk sericin decreases the inflammatory reaction by suppressing cyclooxygenase 2 (COX-2) and nitric oxide genes in the inflammation process. This effect is concentration-dependent [154]. Silk sericin nanoparticles also decrease paw edema induced by carrageenan injection, decreasing the amount of cellular infiltration and the inflammation reaction [155].

2.4.7.4 Moisturizing effect

The main amino acid of silk sericin is serine which is a moisturizing acid. Serine can preserve skin moisture by generating a film on the surface of skin [145]. It was reported that 2% silk sericin gel can decrease transepidermal water loss and improve the smoothness of the skin [156].

2.4.7.5 Biological safety

Sericin is not toxic to cells [10]. However, it was found that sericin at high concentrations (more than 100 μg/mL) may decrease cell availability [147]. When sericin material was implanted subcutaneously in rats, the local effect in terms of the infiltration of inflammatory cells, necrosis, fibrosis, and neovascularization showed that it was a non-irritating material [12, 13].

The advantages (in terms of wound healing promotion, antibacterial and anti-inflammation properties, and safety) and disadvantages of these animal-derived

products and extracts are demonstrated in Table 11. Because of the high efficacy, high safety level, and few adverse effects of silk sericin, it was incorporated into the biocellulose dressing for this study.

Table 3 Characteristics of α-Chitin , β-Chitin, and γ-Chitin [74, 157]
(+, ++, +++ indicate the extent of activity from low to high)

Chitin	Sources	Molecular arrangement	Reactivity for chemical and enzymatic transformation	Thermal degradation	Solubility in common solvent	Chitosan viscosity [160]	Chitosan Molar Mass [160]
α - Chitin	Crab tendons, Shrimp shells, Lobster, Krill, Insect cuticle, Fungal and yeast cell wall	Two antiparallel molecule per unit cell with inter and intra-sheet hydrogen bonds in crystal structure	+	+	+	+++	+++
β - Chitin	Squid pens, Pogonophoran tubes, Vestimetiferan worms tubes	One parallel molecule per unit cell with intra but without inter-sheet hydrogen bonds in crystal structure	+++	+++	+	+	+
γ- Chitin	Cocoon fibers of the *Ptinus* beetle Stomach of *Loligo* squid	Two antiparallel molecule and one parallel molecule per unit with inter and intra-sheet hydrogen bonds in crystal structure (like α –Chitin than β – Chitin)	++	++	+	++	++

Table 4 *In vivo* test and clinical studies of topical chitosan treatment

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [161]	Open dorsal skin wound of beagles	24 wounds (Sample 12, control 12)	Chitosan cotton fiber	Bandage without chitosan	- Granulation tissue of full thickness layer was significantly elevated in chitosan-treated wound after 6, 9 and 15 incision day comparing with control group. - Chitosan could accelerate the proliferation of fibroblasts and the production of type III collagen.	No report

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [87]	Surgical wound of rabbits (0.3 cm thickness from coat to muscle, 2 cm diameter)	24 wounds (Sample 12, control 12)	Chitosan-gelatin sponge dressing	Vaseline sterile gauze	- Wound area of chitosan-treated group were lower than the control (0.8±0.3 VS 1.1±0.3 cm^2, respectively)	No irritation after 48 hours in both group
In vivo test [89]	Full thickness wound of mice	16 wounds (Sample 8, control 8)	Chitosan hydrogel	Without any treatment	- Wound healing time and wound size of chitosan-treated group were lower than the control. - Granulation tissue formation and epithelialization of chitosan-treated group were significant higher than control group.	No toxicity in chitosan-treated group

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [162]	Second degree burn wound of rats	24 wounds (Sample 12, control 12)	Chitosan gel incorporating EGF (10 µg/ml)	EGF gel	- Epithelialization rate of the wound treated with chitosan gel incorporating EGF was significant higher than that of the EGF gel-treated group after 14 days.	No report
In vivo test [163]	Acute wound of rats	12 wounds (Sample 6, control 6)	Chitosan nanofiber mats	Gauze	- Chitosan nanofiber mats showed the faster wound size reduction than gauze dressing at first week after tissue damage.	Chitosan was non- toxicity to human fibroblast cells

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [164]	Burn wounds of rats	30 wounds (6 wounds in each group)	Chitosan gel (MW = 2,000,000, 750,000, 70,000)	2% fucidin ointment (Positive control), Gel without chitosan (Negative control)	- The treated with high molecular weight chitosan (MW = 2,000,000) topical gel showed the highest extent of epithelial and granulation tissue. - The treated with high molecular weight chitosan (MW = 2,000,000) topical gel had fastest wound closure when compared with that treated with the low molecular weight chitosan (MW =70,000) gel and gel without chitosan	No report
Randomized controlled trial [165]	Skin graft donor site patients	20 wounds (half sample and half control)	Chitosan mesh membrane	Bactigras®	- Chitosan mesh membrane showed positive effect on the re-epithelialization and regeneration of the granulation layer than the control. - Pain and itching score in chitosan mesh membrane-treated group were less than control.	No side effect was observed in both groups

Table 5 Type, properties and limitations of collagen obtained from different sources

(+, ++, +++ indicate the extent of activity from low to high)

Collagen sources	Most collagen types	Properties							Limitations	Cost
		Quality for skin treatment	Cell binding property	fibroblast viability	Biocom-patibility	Antigenicity	Denaturation temperature (Td)			
Bovine (Achilles tendon)	I, III	++	++	++	++	+	37°C		- Allergic report around 3% [166] - Concerning about Bovine spongiform encephalopathy (BSE) or Transmissible spongiform encephalopathy (TSE) [104] - Forbidden in some culture	+

Collagen sources	Most collagen types	Properties						Limitations	Cost
		Quality for skin treatment	Cell binding property	fibroblast viability	Biocom-patibility	Antigenicity	Denaturation temperature (Td)		
Porcine (Skin)	I, III	++	++	++	++	+	37°C	- Integration of porcine collagen into scar tissue [104] - Concerning about zoonosis contamination [166, 167] - Forbidden in some religion and culture	+
Marine (Bone, skin, fin, scales)	I	+++	++	+++ [168]	++	+	18-30°C [169]	- Cross reactivity among fish gelatin [170] - Lower yield obtained (12 g of collagen per1 kg of raw material [166] - Low denaturation temperature	++

Table 6 *In vivo* test and clinical studies of topical collagen treatment

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [171]	Diabetic wounds of mice	17 wounds (Sample 9, control 8)	Oxidized regenerated cellulose/collagen plus gauze dressing	Gauze dressing	- The diabetic wound size of collagen-treated group was statistically smaller than control group after 7, 10, 14 days.	No report
Case report [172]	Ulcerative necrobiosis lipoidica diabeticorum	1 wound	Topical collagen gel	-	- Granulation tissue increased in the wound after 6 weeks. - Re-epithelialization was found in the wound edge after 6 weeks.	No report

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
Case series [103]	Chronic wounds with non-responding to other treatments	20 patients with 21 wounds	Bovine-derived 100% native collagen	-	- Wound closure was found 15 of 21 cases after 90 days.	Dermatitis 3 cases
A retrospective study [173]	Chronic wounds	120 patients (Sample 60, control 60)	Collagen dressing	Conventional dressing (silver sulfadiazine, nadifloxacin, povidone iodine, or honey)	- Percentages of healing of collagen-treated group was not significantly higher than conventional-treated group (87% VS 80%) after 8 weeks. - Healthy granulation tissue increasing of collagen-treated group was faster than conventional-treated group. - The conventional-treated group significantly required more treatment of partial split thickness skin graft.	No report

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
Clinical study [174]	Donor site wounds	8 patients with 16 wounds (Sample 8, control 8)	Collagen-based dressing, (SkinTemp ™)	- Fine-mesh gauze dressing (Xeroform™)	- Healing time of collagen-treated group was shorter than control group (7.75 VS 10.62 days). - Pain score of collagen-treated group was less than control group.	No report
Randomized controlled trial [175]	Venous leg ulcers	73 patients (Sample 37, control 36)	55% collagen dressing (Promagan®)	Non-adherent dressing (Adaptic®)	- The amount of healing wound of collagen-treated group was higher than control group (41% VS 31%, $p = 0.079$).	No severe local side effect in both groups

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
Randomized controlled trial [176]	Chronic diabetic neuropathic foot ulcers	124 patients (FCG 33, GAM501 77, control 19)	Formulated Collagen Gel (FCG) and FCG with Ad5PDGF-B (GAM501)	Moist dressing	-Incidence of complete closure of FCG group and GAM501 group were higher than control (45%, 41% VS 31%) - After 1 week, wound radius (mm/week) of both FCG (-0.08 ± 0.61 to 1.97 ± 1.77, $p < 0.002$) and GAM501 (-0.02 ± 0.58 to 1.46 ± 1.37, $p < 0.002$) significantly increased healing rates comparing before treatment. - After 1 week, control group had no significant effect on change in wound radius (mm/week) comparing before treatment (-0.06 ± 0.32 to 0.78 ± 1.53, $p > 0.05$).	Collagen was safe for wound healing

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
Randomized controlled trial [100]	Pressure ulcers	65 patients (Sample 35, control 30)	Topical collagen	Hydrocolloid dressing	- Percentage of complete healing of collagen-treated group was not significantly higher than control group (51% VS 50%) after 8 weeks. - Mean healing time of collagen-treated group was not significantly higher than control group (5 VS 6 weeks).	No adverse event in both groups
Randomized controlled trial [177]	Diabetic foot ulcers	75 patients (Sample : control ratio 2:1)	Collagen-alginate topical wound dressing	Gauze moistened with normal saline	- Percentage of complete healing of collagen-treated group was higher than control group (48% VS 36%). - The mean percent reduction of the wound area of collagen-treated group was higher than control group (80.6 ± 6% VS 61.1 ± 26%, $p > 0.05$)	Collagen was safe for wound healing

Table 7 Clinical studies of topical honey treatment

Study design	Wound types	Number of subjects	Honey type	Control treatment	Outcomes	Side effects
Randomized controlled trial [178]	- Shallow wounds (deeper ≤ 2 cm, size ≤ 100 cm^2) - Abrasions wound (size 10 -100 cm^2)	87 (Sample 40, control 42, dropped out 5)	Natural honey	IntraSite gel® (amorphous hydrogel contain propylene glycol)	- Healing times of both groups were not significantly different. - Satisfactions of both groups were not significantly different. - Honey was low average cost of treatment per patient than IntraSite® Gel (R0.49 VS R12.03).	Itching: Honey 27%, IntraSite gel® 31% Pain: Honey 10% Short time burning: Honey 2 case
Randomized controlled trial [179]	Partial-thickness burns of less than 40% of TBSA (Total body surface area)	100 (Sample 50, control 50)	Natural honey	Mafenide acetate	- Epithelialization of honey-treated group was significantly higher than the control (100% VS 84%, respectively, after 21 days). -Amount of bacterial reduction of honey-treated group were higher than control (45 VS 32, respectively, after 21 days).	No irritation, allergy, or other side effects in both groups

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
Randomized controlled trial [180]	First and second degree of burn of less than 50% of TBSA	78 (Sample 37, control 41)	Natural honey	Silver-sulfadiazine (SSD)	- Healing times of honey-treated group were less than the control (18.16 VS 32.68 days, respectively). - Bacterial reduction rate of honey-treated group was faster than control. - Percentage of complete healing wounds of honey-treated group were significantly higher than the control (81% VS 37%, respectively).	No report
Randomized controlled trial[181]	Chronic wounds	42 (Sample 22, control 20)	Natural honey	Povidone-iodine dressing	- Percentage of complete healing wounds of honey-treated group were higher than the control (31.82% VS 0%, respectively, after 6 weeks). - Wound surface area and pain score in honey-treated group were significantly less than the control group.	Honey: no adverse skin reaction Povidone-iodine dressing: 2 adverse skin reactions

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
Randomized controlled trial [182]	Split-thickness skin graft donor site	100 (Sample 50, control 50)	Natural honey	Vaseline gauze	- Percentage of complete healing wounds of honey-treated group were significantly higher than the control (100% VS 76%, respectively, after 10 days). - No bacterial growth was found in both groups. - Pain in honey-treated group was not significantly less than the control group.	No allergic reaction in both groups
Randomized controlled trial [183]	Neuropathic diabetic foot ulcers	63 (Sample 32, control 31)	Manuka honey	Saline-soaked gauze	- Healing times of honey-treated group were significantly less than the control group (31±4 VS 43±3 days, respectively). - Number of patients required antibiotic treatment of honey-treated group was none but that of the control group was 29%.	No report

Table 8 *In vitro* test and *in vivo* test of topical anabolic steroid treatment

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vitro test [184]	Fibroblast cells	6 samples	Porcine placental extracts (30 mg/ml)	0.5% Fetal bovine serum (FBS)	- Proliferation of fibroblast cells cultured in porcine placental extracts (30 mg/ml) was significantly higher than control.	No report
In vivo test [184]	Diabetic wounds of rats	144 wounds (Sample 72, control 72)	Porcine placental extracts	Normal saline, No treatment	- TGF-β1 and bFGF levels in wound skin of rat which was treated with porcine placental extracts were significantly greater than control at day 5 to 15 after burns. - Healing time of the wound treated with porcine placental extracts was significantly less than that of control group.	No report

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [119]	Full thickness wound of rats	60 rats (Sample 30, control 30)	Cream + porcine testis-extracted steroid	Cream without porcine testis-extracted steroid	- The healing efficiency in terms of collagen formation and tensile strength of the wound treated porcine testis-extracted steroid was higher after 7 days of treatment	No skin rash and toxicity in both groups

Table 9 *In vivo* test of peptide treatment

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [129]	Full thickness wound of mice	10 rats (20 wounds) (Sample 10, control 10)	Tylotoin extracted from salamander at concentration 20 µg/ml	Vehicle of extract	- Topical treatment with tylotoin extracted from salamander at concentration 20 µg/ml accelerated full thickness wound healing process in mice. - After 10 days, the wound areas of tylotoin-treated group were almost completely closed but the wound areas of the control group were closed around 74%. - Tylotoin increased proliferation and migration of keratinocyte, fibroblast and vascular endothelial cells.	No report

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [185]	Full thickness wound of mice	48 wounds (Sample 24, control 24)	AH90 ointment (1 mg raw extracted and ultra-filtrated ointment)	Ointment without AH90 peptide and without treatment	- AH90 ointment significantly increased percentage of wound closure in rats comparing with control after 2, 4 and 6 days' post-injury. - Fibroblast cells and collagen production of the wound treated with AH90 peptide were also significantly higher than those of the control group after 2, 4, and 6 days' post injury.	No report

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [186]	Full thickness wound of mice	18 wounds (Sample 6, control 12)	Lyophilized frog skin ointment	Ointment without peptide and without treatment	- Frog skin ointment significantly increased percentage of wound closure in rats comparing with control after 4 and 6 days' post-injury. - Number of inflammatory cells of frog skin ointment-treated group was significantly lower than control group after 2, 4 and 6 days' post-injury. - Fibroblast cells and collagen production of the wound treated with frog skin ointment were significantly higher than those of the control group after 6 days' post injury.	No report

Table 10 *In vivo* test and clinical studies of topical silk sericin treatment

Study design	Wound types	Number of subjects	Study group	Control treatment	Outcomes	Side effects
In vivo test [187]	Corneal abrasion of rats	3-5 independent corneas in each group	Silk sericin solution at 1%, 5% and 10%	Saline solution	- Silk sericin in all concentration had higher rate of wound healing than control group. - High concentration of silk sericin could accelerate corneal healing better than low concentration. - The corneal healing rate constant of rat eyes instilled 10% silk sericin solution was approximate 12-fold that of rat eyes instilled with saline solution.	No report
In vivo test [15]	Full thickness wound of rats	18 rats (36 wounds) (Sample 18, control 18)	8% silk sericin cream	Povidone iodine solution and cream base without silk sericin	- The percentage of wounds size reduction of silk sericin cream-treated group was significantly higher than cream base-treated group after 15 days but was not significant difference comparing with povidone iodine-treated group. - Inflammatory reaction of silk sericin cream were lower than control group	No toxicity in both groups

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [188]	Full thickness wound of rats	24 rats (48 wounds) (Sample 18, control 18)	Genipin cross linked silk sericin / polyvinyl alcohol (PVA) scaffolds	Genipin cross linked PVA scaffolds without silk sericin	- Percentage of wound size reduction of genipin cross linked silk sericin / PVA scaffolds –treated group was significantly higher than control group. -The number of macrophages of genipin cross linked silk sericin / PVA scaffolds –treated group was significant lower than control group after 7 days. - The extent of type III collagen in wounds of genipin cross linked silk sericin / PVA scaffolds – treated group was higher than control group.	No report

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
In vivo test [189]	Full thickness wound of rats	24 rats (48 wounds) (Sample 18, control 18)	Ethyl alcohol-precipitated silk sericin /PVA scaffolds	Ethyl alcohol-precipitated PVA scaffolds without silk sericin	- Percentage of wound size of the silk sericin dressing group was significant lower than control group after 14 days. - Percentage of type III collagen formation of genipin cross linked silk sericin / PVA scaffolds – treated group was higher than control group	No report
Randomized controlled trial [17]	Split-thickness skin graft donor site	30 patients (half with sample and another side with control)	Ethyl alcohol-precipitated silk sericin /PVA scaffolds	Bactigras® (chlorhexidine paraffin gauze dressing)	- Healing time of the wound treated with 3% silk sericin was significantly less than that of the control group (12±5 VS 14±5.2 days, respectively). - Pain score of the wound treated with 3% silk sericin was lower than that of the control group from day 1-5.	No severe adverse reaction in both groups

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
Randomized controlled trial [190]	Second degree burn wounds	29 patients (Sample 15, control 14)	Silver zinc sulfadiazine cream containing 8% silk sericin	Silver zinc sulfadiazine cream	- Healing time of the wound treated with silver zinc sulfadiazine cream containing 8% silk sericin was significantly less than that of the control group (22.42±6.33 VS 22.98±9.27 days, respectively). - Percentage of wound size reduction of the wound treated with silver zinc sulfadiazine cream containing silk sericin -treated group was significantly higher than that of the control group after 14 and 42 days. - Pain score of both groups were not significant difference. - No infection was found in both groups.	No severe adverse reaction in both groups

Study	Wound types	Number of subjects	Study group	Control group	Outcomes	Side effects
Randomized controlled trial [41]	Split-thickness skin graft donor site	30 patients (Split wound and treated one side with sample and another side with control)	Bilayered wound dressing containing1% silk sericin	Bactigras®	- Healing time of the wound treated with 1% silk sericin was significantly less than that of the control group (11±6 VS 14±6 days, respectively). - Pain score of the wound treated with 1% silk sericin was significantly lower than that of the control group from day 1-5. - Transepidermal water loss index of the wound treated with 1% silk sericin was lower than that of the control group after healing days. - No sign of infection was found in both groups.	No systemic adverse reaction in both groups

Table 11 Advantages and disadvantages of animal-derived products in topical wound application

(+ ; positive activity, ± ; may have positive or negative activity)

Animal-derived products	Advantages				Disadvantages
	Wound healing promotion	Anti-bacteria	Anti-inflammation	Safety	
Chitosan	+	+		+	- Molecular weight has significant effect on wound healing properties. - Allergy in patients who are allergic to arthropods including shrimp, crab, insects, and shell fish
Collagen	±			+	- Fast degradation rate - Concerning zoonosis contamination - Case report of allergic reaction to bovine collagen - Expensive
Honey	+	+	+	+	- Antibacterial property can be decreased when in contact to wound exudate except manuka honey. - Pain in sensitive nerve ending patients - local atopic reaction
Anabolic steroids	+			+	- Absorbed to blood circulation, leading to systemic side effect

Animal-derived products	Advantages				Disadvantages
	Wound healing promotion	Anti-bacteria	Anti-inflammation	Safety	
Peptide	+			+	- Limited research document
Proteoglycan	+			+	- Poor wound healing property at high concentration. - Limited research document
Sericin	+	±	+	+	- Cell viability is decreased at high concentration - Antimicrobial activity depends on degumming process

2.5 Topical antimicrobial agents and dressing

Wound infection is an important cause of non-healing wounds. The risks of wound infection depend on many factors including wound size or depth, wound environment, cleaning, and the treatment process. Microorganisms including aerobic Gram-positive bacteria, aerobic Gram-negative bacteria, and anaerobic bacteria infect the wound and produce protease enzymes to destroy growth factors and proteins. They consume nutrients and oxygen from the wound. They also stimulate a human immune response to release inflammatory mediators that interfere with fibroblast production and the wound healing process. Infected wounds lead to unexpected pain, malodor, and excessive discharge. Moreover, wound infection may lead to osteomyelitis and sepsis by transfer of the infection from the skin to bone and blood. Bacteria can protect themselves from some antimicrobial agents with bacterial resistance processes including efflux pumps. Finding antimicrobial agents for wound treatment with high efficacy and fewer adverse effects is of great interest.

Topical antimicrobial agents and dressings should have a broad spectrum of action and be able to penetrate the eschar. They should release a high concentration of antibacterial agent at the site of the infection [158]. Using topical antimicrobial agents and dressings has many advantages including the avoidance of systemic toxicity and side effects, and decreased induction of bacterial resistance [159]. However, they have local side effects such as allergic contact dermatitis and lack of depth penetration. Advantages and disadvantages of topical antimicrobial dressings are shown in Table 12.

Table 12 Advantages and disadvantages of topical antimicrobial agents and dressings

Advantages [160-162]	**Disadvantages** [162-164]
• Localized antimicrobial agent at the site of infection • Limited amount of antimicrobial agent • High concentration at the site of infection • Contact directly with microorganism • New antimicrobial agents that do not have systemic antimicrobial form • Easy to use • Good compliance for patient • Minimize toxicity and reduce multi-drug resistant microorganisms from systemic antimicrobial agents	• Few topical antimicrobial agents are effective in randomized clinical trials • Cannot treat severe or deep wound infections • Difficult to specify dose with certainty • May absorb systemically if applied to large wounds • Affect the healing process • May irritate or cause hypersensitivity

Ideal topical antimicrobial properties:

- Broad-spectrum antimicrobial agent
- Rapid action
- Long duration of action
- Do not induce drug resistance
- Good distribution
- Low systemic absorption
- Safe and biocompatible

2.5.1 Topical antimicrobial agents

Topical antimicrobial agents for wound treatment have various forms such as creams, ointments, gels, or dressings. They are separated into two groups, antiseptic and antibiotic. Most antibiotics are produced from microorganisms but antiseptics are synthetic agents. They contain bacteriostatic

or bactericidal activity. Antiseptics are different from antibiotics because antiseptics can be transported through the lymphatic system to destroy bacteria within the body. However, applying large amounts of topical antimicrobial agents may be toxic, induce acute inflammation, or decrease collagen. Examples of topical antibiotic agents and antiseptic agents are shown in Tables 13 and 14.

Table 13 Topical antibiotic agents' documents

Antibiotic agents	Produced from	Mechanism of action	Antibacterial activity	Notification
1. Neomycin [198, 199]	*Streptomyces fradiae*	- Bactericidal - Bind to 30s ribosomal subunit of bacteria to inhibit protein synthesis	Aerobic gram negative bacteria and Staphylococci	- Cannot be against to other gram positive cocci and less active against anaerobic bacteria - High drug resistance such as Staphylococci, *Escherichia coli, Proteus* and *Klebsiella* - Maybe cause of contact dermatitis or skin damage
2. Gentamicin [199]	*Micromonospora purpurea*	- Bactericidal - Bind to 30s ribosomal subunit of bacteria to inhibit protein synthesis	- Gram negative bacteria including *Pseudomonas aeruginosa* - Some gram positive bacteria such as Staphylococci, Streptococci	- Need to use for 3-4 times per day - Resistance and super-infection developed rapidly - Cautions when use in burn wound because it is rapid absorption associated with ototoxicity and nephrotoxicity

Antibiotic agents	Produced from	Mechanism of action	Antibacterial activity	Notification
3. Polymyxins [195]	*Bacillus polymyxa*	- Bactericidal - Disturb bacterial membrane	Some aerobic gram negative bacteria including *Pseudomonas aeruginosa*	- Cannot be against to gram positive bacteria - Rarely cause of dermatitis - Increasing level of microorganism resistance relate to dose
4. Bacitracin [195, 198]	*Bacillus subtilis*	- Bactericidal - Interrupt the dephosphorylation of molecule that is component of bacterial cell wall	Gram positive bacteria	- A few reports about drug resistance and side effects. - Low toxicity and inexpensive

Antibiotic agents	Produced from	Mechanism of action	Antibacterial activity	Notification
5. Mupirocin [199]	*Pseudomonas fluorescens*	- Bacteriostatic at low concentrations and bactericidal at high concentrations - Reversible inhibition of isoleucyl-tRNA synthesis so disruption protein and cell wall synthesis	Gram positive bacteria	- Cannot be against to *Pseudomonas aeruginosa* and Enterococci - Often used to treat impetigo cause from *Staphylococcus aureus* or *Staphylococcus pyrogen* and nasal colonization of MRSA - Less toxicity - Cross drug resistance did not occur but it is used for long time may be induced drug resistant Staphylococci.
6. Fusidic acid	*Fusidium coccineum*	- Bacteriostatic - Binds to elongation factor G (EF-G) and the ribosome result in inhibiting further bacterial protein synthesis	- Gram positive bacteria *Staphylococcus aureus*, Streptococci, Corynebacteria and Clostridia	- May increase the risk of developing antibiotic resistance - Some report about drug resistance

Antibiotic agents	Produced from	Mechanism of action	Antibacterial activity	Notification
7. Mafenide acetate [200-202]	Synthesized	- Bacteriostatic - Interrupt to bacterial folic acid metabolism	- Gram positive bacteria including *Clostridrium spp.* - Gram negative bacteria including *Pseudomonas* species	- Cannot be against to MRSA - Good penetration to eschars - More appropriate for non-facial burns - Side effects such as maculopapular rash, contact dermatitis - Metabolized to a carbonic anhydrase inhibitor result in metabolic acidosis -The large amount of absorption leads toxicity such as pulmonary function

Table 14 Topical antiseptic agents' documents

Antiseptic agents	Mechanism of action	Antibacterial activity	Notification
1. Hydrogen peroxide [59, 198]	- Bactericidal - DNA damage - Inactivation of enzyme by producing free radical	- Gram positive bacteria - Gram negative bacteria	- 3% Hydrogen peroxide solution is most often used to clean wound - Inexpensive - May be irritate or directly toxic to keratinocyte to the skin
2. Chlorhexidine [203]	- Bacteriostatic at low concentrations and bactericidal at high concentrations - Cationic molecule to negatively charged bacterial cell walls	- Gram positive bacteria - Gram negative bacteria	- Long duration - Slow activity - More hypersensitivity and dangerous to the eyes and ears - Very painful
3. Triclosan [198]	- Bacteriostatic at low concentrations and bactericidal at high concentrations - Inhibit of fatty acid synthesis lead to damage cell membranes	- Gram positive bacteria - Gram negative bacteria	- Report about *Escherichia coli* resistance - Concerned about other species resistance in the future because of widespread using of this agent

Antiseptic agents	Mechanism of action	Antibacterial activity	Notification
4. Iodine (Povidone iodine) [203]	- Bactericidal - Destroy protein, fatty acid and DNA of microorganism	- Gram positive bacteria - Gram negative bacteria - Biofilm	- Iodine absorption result in renal and thyroid dysfunction [204] - Povidone iodine is one form of iodine that less activity than controversy iodine so it is action by longer contact to the wound about 2 minutes. - Povidone iodine is less toxic or irritate to skin and resistant report has not been documented. - New form of iodine is cadexomer which is sustain release and no toxic to tissue

Antiseptic agents	Mechanism of action	Antibacterial activity	Notification
5. Silver (Silver nitrate, silver sulfadiazine, Nanosilver) [196, 204]	- Bactericidal - Destroy cell wall, cell membrane, and enzyme - Block DNA replication - Interrupt bacterial respiration and adenosine triphosphate synthesis	- Gram positive bacteria - Gram negative bacteria - Biofilm	- Short duration (repeated every 4 hours for silver nitrate, every 12 hours for silver sulfadiazine) - Rapidly inactive when contact with discharge of the wound - Cytotoxic to host cells in vitro - Side effect such as neutropenia, leukopenia, pseudoeschar, yellow-gray skin, and irritation - Sustain release silver such as nanosilver decreases frequency of using and has fewer side effects. - Hyponatremia and hypochloremia may be found when is used in large wound [49]. - Silver resistance shows in some report such as *Escherichia coli*, *Enterobacter cloacae*, *Klebsiella pneumoniae*, *Acinetobacter baumannii*, *Salmonella typhimurium* and *Pseudomonas stutzeri* [205]

Antiseptic agents	Mechanism of action	Antibacterial activity	Notification
6.Polyhexamethylene biguanide (PHMB) [193]	- Bacteriostatic at low concentrations and bactericidal at high concentrations - Binding between negative charged phosphate head group of bacteria cell wall and cationic group of PHMB - Promoting interaction of hexamethylene spacer groups with hydrophobic interior of the membrane bilayer causing membrane fluidity and permeability interfering and cell death	- Gram positive bacteria - Gram negative bacteria	- Should cover at the wound at least 5-15 minutes - Safety and biocompatibility to skin - May not be suitable for treatment cartilage and central nervous system because of incompatibility and toxicity - No report of resistance

2.5.2 Topical antimicrobial dressings

Size, depth, patient sensitivity, allergy, and the amount of exudate are important factors to be considered in selecting the appropriate dressing for a wound [42]. Advantages and disadvantages of topical antimicrobial wound dressings are shown in Table 15. Because of the high efficacy, high safety level, and few adverse effects of PHMB, it was incorporated with silk sericin into the biocellulose dressing for this study.

Table 15 Advantages and disadvantages of topical antimicrobial wound dressings

Antimicrobial wound dressings	Components	Advantages	Disadvantages
1. Silver dressing			
1.1 Acticoat™	- Silver nanoparticle coated polyethylene mesh	- A broad spectrum antibacterial - Long acting (reduce wound dressing change) [206]	- Cannot absorb wound exudate (inappropriate to high exudate) - Need secondary dressing - Painful - High cost - Report adverse effects: re-epithelialization impairment, bone marrow toxicity, leukopenia, and color of the wound changing - Bacterial resistance report [206]

Antimicrobial wound dressings	Components	Advantages	Disadvantages
1.2 Aquacel® Ag	- Hydrofiber wound dressing containing silver ion	- For moderate to high exudate - A broad spectrum antimicrobial - Prolong using to 7 days - Easy for application and conformability [34]	- The excess absorption leads to wound dehydration - High cost - Report adverse effects: re-epithelialization impairment, bone marrow toxicity, leukopenia, and color of the wound changing - Bacterial resistance report [206]
1.3 Urgotul® SSD	- Polymer mesh impregnated with carboxymethyl cellulose, petroleum jelly and silver sulfadiazine	- For low exudate wound - A broad spectrum antimicrobial - Non-adherence, easy for removal [44, 207]	- High cost - Report adverse effects: re-epithelialization impairment, bone marrow toxicity, leukopenia, and color of the wound changing - Bacterial resistance report [206]

Antimicrobial wound dressings	Components	Advantages	Disadvantages
2. Chlorhexidine dressing			
2.1 Bactigras®	- Gauze of leno weave impregnated with a chlorhexidine acetate 0.5% in white soft paraffin	- Both bacteriostatic and bactericidal - A broad spectrum antimicrobial [208] - Low cost	- Low antibacterial activity for *Pseudomonas aeruginosa* [209, 210] - Could not exhibit bactericidal activity more than a 4 log reduction of *Escherichia coli* [210] - Does not have moist wound keeping property - Cannot absorb wound exudate - Painful
3. Polyhexamethylene biguanide dressing			
3.1 Suprasorb X+PHMB bacterial cellulose	- PHMB-containing bacterial cellulose dressing (0.3% PHMB)	- A broad spectrum antimicrobial - Biocompatibility with human cell - Low adsorption - No known of bacterial resistance - Good clinical tolerability - Low risk of contact sensitization	- May not be suitable for treatment cartilage and central nervous system because of incompatibility and toxicity

Antimicrobial wound dressings	Components	Advantages	Disadvantages
3.2 Excilon™ AMD sponge	- Sponges impregnated with PHMB (0.2% PHMB)	- A broad spectrum antimicrobial - Biocompatibility with human cell - High adsorption - No known of bacterial resistance - Good clinical tolerability - Low risk of contact sensitization	- The excess absorption leads to wound dehydration - May not be suitable for treatment cartilage and central nervous system because of incompatibility and toxicity

2.6 Polyhexamethylene biguanide

PHMB is a synthetic compound. Its structure is similar to that of the natural antimicrobial peptides (AMPs) of keratinocytes and inflammatory neutrophils [165]. PHMB was approved as a medicinal product in the 1990s [18]. It is a bacteriostatic if administered at low concentrations (1–10 mcg/mL) and bactericidal at high concentrations (≥ 10 mcg/mL) [44]. It is a broad-spectrum antimicrobial agent with high efficacy and low toxicity [20]. It can act as a biocide against aerobic and non-aerobic bacteria through binding between the negatively charged phosphate head groups of the bacterial cell wall and the cationic group of PHMB, and it promotes interaction of hexamethylene spacer groups with the hydrophobic interior of the membrane bilayer, leading to membrane fluidity and permeability interference, and cell death [19]. However, this interaction is rarely found in human and animal cells. Ikeda *et al* found that PHMB could interact with the phosphatidylglycerol of bacterial cells and a mixture of phosphatidylglycerol and phosphatidylcholamine (PC) but had very little effect on the neutral lipids PC, a principal phospholipid of mammalian cells, and phosphatidylethiolamine (PE), a principal phospholipid of bacteria [166, 167]. Moreover, PHMB can bind to DNA and other nucleic acids of bacteria. The intra-cation spacing in PHMB is optimized for bacterial DNA binding. Strong complex formation of PHMB and bacterial DNA was performed at 0.25 mM [168]. The minimal microbial PHMB concentration for *S. aureus* was 0.1 mcg/mL, for *Bacillus subtilis* 0.5 mcg/mL, *E. coli* 0.5 mcg/mL, and *P. aeruginosa* 25 mcg/mL [22]. Because of its high tolerability and biocompatibility, PHMB is preferable for chronic wound treatment compared to chlorhexidine and povidone iodine [169]. It can maintain its activity for 14 days. Low toxicity is one of the advantages of PHMB. Mild irritation to skin and no significant cancer induction have been reported in humans. No evidence of the development of resistance has been reported [170]. The mechanism of bacterial resistance, efflux pumps, cannot remove it [160]. Therefore, many wound treatment products are composed of PHMB.

2.6.1 Physical and chemical properties of PHMB

The molecular weight of PHMB depends on the degree of polymerization, and is around 5,049 g/mol at 12 degrees of polymerization

[171]. Its viscosity is close to that of water. The terminal groups of PHMB are amine and cyanoguanidine, which are unlikely to be biodegraded [172]. It has a molecular arrangement as micelles in which hydrophobic methylenic segments point toward the center of the sphere and hydrophilic segments point toward the outside of the sphere. Therefore, it has high solubility in agents with high polarity and hydrogen-bonding tendency. PHMB is soluble in both water (more than 40% w/w) and methanol, with chemical stability but poor solubility in ethanol. Sunlight, water temperature, and pH fluctuation rarely have effects on PHMB stability. Maximum activity is found at pH 5–6 [22]. However, at a temperature more than 463 K, PHMB chain degradation was reported. Aqueous solution of PHMB is absorbed at a UV wavelength of 236 nm.

2.6.2 Safety and efficacy of PHMB

PHMB is an antimicrobial agent which has many efficacies [23] including broad-spectrum antimicrobial agent, biocompatibility with human cells, low adsorption, no known bacterial resistance, good clinical tolerability, and low risk of contact sensitization. Therefore, there are several applications of PHMB [171] such as sanitizer in the textile industry and toilets, disinfectant for medical, dental, and farm equipment, ingredient in hand washes and rubs, air filter treatment, water treatment, swimming pools, and hot tubs, preservative in cosmetics, fabric softeners, contact lens solution, and leather. For medical products, PHMB are used as antimicrobial agent in wound irrigation (solution or gel) such as Prontosan® solution (0.1% PHMB), Lavasorb® solution (0.02/0.04% PHMB), and Lavanid® wound gel (0.04% PHMB). PHMB is also composition in antimicrobial dressings such as Telfa™ AMD (0.2% PHMB), Kerlix™ AMD gauze (0.2% PHMB), Excilon™ AMD sponge (0.2% PHMB), Suprasorb® X+PHMB biocellulose (0.3% PHMB), and Kendall AMD foam (0.5% PHMB).

The duration of topical antiseptic treatment is usually 2–5 days and in general should not exceed 14–21 days. It should cover the wound for at least 5–15 min.

Dressings containing 0.2% PHMB decreased bacteria in the wound and prevented wound infection, while bacterial resistance was not observed in long-term use [170]. In an *in vitro* study, a low concentration of PHMB (0.5 mcg/mL) accelerated wound healing using a wound model of respiratory ciliary epithelial cells after 96 h [173]. Muller and Kramer found that PHMB at a low concentration (0.005%) did not increase the catabolism of PGs in an *ex vivo* test [174]. *In vivo* and clinical studies are shown in Table 16.

Table 16 *In vivo* test and clinical studies of PHMB dressing treatment

Study design	Wound types	Number of subjects	Study group	Control treatment	Outcomes	Side effects
In vivo test [221]	Partial-thickness wounds infected *Pseudomonas aeruginosa* of pigs	3 pigs (18 wounds) (Sample 18, control 18)	Gauze impregnated 0.2% PHMB	Gauze without an antibacterial agent	- PHMB dressing significantly reduced by 4 to 5 logs the amount of *P. aeruginosa* at the wound bed. - PHMB dressing significantly able to reduce the inoculum within the dressing itself at 24 and 48 hours' post-inoculation compared to control.	No report

Study design	Wound types	Number of subjects	Study group	Control treatment	Outcomes	Side effects
A retrospective study [222]	Venous leg ulcers	112 wounds (Sample 59, control 53)	PHMB solution	Ringer's solution or saline solution	- PHMB solution cleansing group had less healing time than control group. - Percentage of healing wound of PHMB-treated group was significantly higher than control group after 6 months (97% VS 89%). - Infection of PHMB-treated group was less than control group (3% VS 13).	No report
Clinical study [223]	Second degree burn wounds	4 patients 4 wounds (Divided into 3 zone)	0.04% PHMB solution	Undiluted povidone-iodine solution or 1% silver nitrate solution	- The wound areas that treated with 0.04%PHMB solution showed the best re-epithelialization than control group after 7 days. - The necrosis zone of PHMB-treated group was less than control group. - No case of wound infection occurred.	No side effect or adverse reactions were observed in PHMB group.

Study design	Wound types	Number of subjects	Study group	Control treatment	Outcomes	Side effects
A cohort study [24]	Non-healing locally infected and/or critically colonized wounds of various etiologies	16 patients	PHMB-containing bacterial cellulose dressing	-	- PHMB dressing reduced biofilm in the wound more than 20 patients (95%) after 24 weeks. - PHMB dressing significantly promoted granulation tissue comparing day 0 (77% VS 38%). - The wounds treated with PHMB dressing were complete epithelialization (75%). - PHMB dressing reduced pain.	No irritation
Randomize control trial in clinical study [25]	Infected wounds	42 wounds (Sample 24, control 18)	PHMB-containing bacterial cellulose dressing	Silver dressing	- PHMB-containing bacterial cellulose dressing was significantly better reduced pain score than control group. - PHMB-containing bacterial cellulose dressing was more rapidly and superior reduced local wound infection than control.	No adverse events in both groups

Study design	Wound types	Number of subjects	Study group	Control treatment	Outcomes	Side effects
Randomize control trial in clinical study [37]	Second degree burn wounds	60 patients 72 wounds (Sample 34, control 38)	PHMB-containing bacterial cellulose dressing	Silver sulfadiazine cream	- PHMB-containing bacterial cellulose dressing were significantly faster and better pain reduction than control group. - The dressing changes, ease of use, and cost saving of PHMB-containing bacterial cellulose dressing group showed better result than silver sulfadiazine cream group	PHMB safe for patients

PHMB may not be suitable for treatment of cartilage and the central nervous system because of incompatibility and toxicity [19]. Intraperitoneal instillation of 0.04% PHMB induced systemic hypotension and local vasodilation in mice [22]. Topical PHMB (0.02%) had an effect on vasodilation in the ear skin of hairless mice [19, 175]. A high concentration of PHMB may irritate the respiratory tract and eyes. However, there was no evidence of oral toxicity to rabbits at 20 mg/kg/day and no long-term dermal toxicity at 150 mg/kg/day [19]. The minimal concentration for skin irritation in rats was over 5%, for eye irritation in rabbits was over 25%, and was less than 1% in humans [22]. The general concentration in wound dressings is around 0.3% PHMB which is safe for skin. Moreover, no development of bacterial resistance of PHMB has been reported [23]. No evidence of carcinogenicity or mutagenicity was found in *in vitro* and *in vivo* studies.

Contraindications of PHMB [23]:

1. Irrigation solution in the peritoneal cavity
2. Antiseptic joint irrigation
3. The entire central nervous system
4. The middle ear or inner ear or intraocular administration

A new development, a PHMB solution combining aqueous PHMB solution and egg phosphatidylcholine as an oil-in-water emulsion or liposomes, could reduce toxicity of PHMB to L929 cells while maintaining an antiseptic effect. It may be used with sensitive tissues such as the peritoneum, mouth, and joint cavities [176].

The combination of silk sericin and PHMB in a biocellulose wound dressing has advantages for STSG donor site wounds because it may absorb wound exudate, have a cooling effect for pain reduction, keep moisture in the dressing, activate collagen synthesis which promotes wound healing, and have an antibacterial effect to protect against infection.

Our preliminary research found the optimal silk sericin and PHMB concentrations could promote collagen type 1 (Appendix H; Figure 2) [177], had antimicrobial activity against *B. subtilis* (ATCC 6633, Gram-positive), *S. aureus* (ATCC 25923, Gram-positive), MRSA (Gram-positive), *E. coli* (ATCC 25922, Gram-negative), *Acinetobacter baumannii* (ATCC 19606, Gram-negative), and *P.*

aeruginosa (ATCC 27853, Gram-negative) (Appendix H; Figure 3) [177], and a non-adhesive effect *in vitro* (Appendix H; Figure 4). The interaction between silk sericin and PHMB was also studied and presented in our previous research [177]. We found that silk sericin and PHMB had a weak interaction which slightly affected the amide I region or the secondary structure of the protein, indicating a change in the secondary structure of the protein. However, this interaction did not affect the activity of silk sericin or PHMB in the dressing. Moreover, interaction between the biocellulose dressing and other molecules (silk sericin and PHMB) was not observed. The structure of the dressing was investigated using scanning electron microscope (SEM). The dressing was an ultrafine network structure. The size of fiber was less than 40 nm and pore size was less than 100 nm (Appendix H; Figure 1). The physical properties of this dressing were moderate wound absorption, no degradation of enzymes, and non-adhesion to the skin [178]. Therefore, it could absorb exudate, did not macerate or split out, stays on the wound, and reduced pain. *In vivo* tests on the efficacy of the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras® on full-thickness skin wounds in 24 rats indicated that the area fraction of collagen, a wound healing acceleration parameter of the biocellulose dressing, was significantly higher than controls on day 14 (Appendix H; Figure 5) [24]. The wound size reduction of wounds treated with the biocellulose dressing was also significantly higher than Bactigras® on day 14, and wounds were completely closed by day 21 (Appendix H; Figure 6) [24]. There was one infected wound in the biocellulose dressing group and four infected wounds in the Bactigras® group. The safety study of the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras® in rats showed that the rats implanted with the biocellulose dressing were healthy and had less irritation and inflammation [24]. However, for materials and methods of some important *in vitro* and *in vivo* tests are shown in Appendix H.

According to the literature reviews and the results of our preliminary study on the advantages of the biocellulose wound dressing containing silk sericin and PHMB, the objective of this study is to investigate the safety of the biocellulose wound dressing containing silk sericin and PHMB in healthy volunteers and the clinical efficacy of this dressing for STSG donor site treatment compared to Bactigras®, the

standard commercially available dressing, for STSG donor site wounds at the King Chulalongkorn Memorial Hospital, in terms of time for complete re-epithelialization and wound quality, which have never been studied before, rate of infection, pain evaluation, and adverse events.

CHAPTER 3 RESEARCH METHODOLOGY

3.1 Study design

Phase I clinical study

Prospective, randomized, controlled matched-pair study.

Phase II clinical study

Prospective, randomized, controlled matched-pair study.

3.2 Sample and population

3.2.1 Target population

Phase I clinical study

Healthy volunteers at the Faculty of Pharmaceutical Sciences, Chulalongkorn University.

Phase II clinical study

STSG donor site wounds from procedures carried out in the Division of Plastic and Reconstructive Surgery, Department of Surgery, King Chulalongkorn Memorial Hospital.

3.2.2 Sample

Phase I clinical study

Healthy volunteers at the Department of Pharmacy Practice, Faculty of Pharmaceutical Sciences, Chulalongkorn University between October 2014 and February 2015.

Phase II clinical study

STSG donor site wounds from procedures carried out in the Division of Plastic and Reconstructive Surgery, Department of Surgery, King Chulalongkorn Memorial Hospital between December 2015 and October 2016.

3.2.3 Sample size calculation

Phase I clinical study

The sample size was calculated following McNamee *et al*. [179]. They found that there should be at least 100 subjects in a wound dressing study for a 99% chance

of having a 5% incidence of adverse skin effects. If 10% of subjects drop out, there should be 112 subjects in this study.

Phase II clinical study

This study investigated two dependent samples to compare the wound healing time (the day the dressing detached by itself with no exudate or air-contact pain) between the biocellulose wound dressing containing silk sericin and PHMB and Bactigras® groups. The sample size was calculated by the following:

$$N = \frac{(Z_\alpha + Z_\beta)^2 S_p^2}{D^2}$$

N	=	sample size
Z_α	=	Z value for alpha error
Z_β	=	Z value for beta error
S_p^2	=	pooled variance
D^2	=	effect size; difference in time taken for complete healing of STSG donor site treated with two types of dressing determined as 2 days

According to the study of Lohsiriwat *et al*. [180] which compared an ionic silver-containing hydrofiber dressing with paraffin gauze dressing for STSG donor wound treatment, the wound healing times were 7.9 ± 2.47 and 11.2 ± 3.52 days, respectively ($p < 0.05$). The primary objective of Lohsiriwat *et al*. study was the wound healing time for STSG donor wound treatment that was the same primary objective as this study. Lohsiriwat *et al*. used an ionic silver-containing hydrofiber dressing as a subject that had characteristics and properties such as absorption capacity and antimicrobial agent composition close to biocellulose wound dressing containing silk sericin and PHMB. Moreover, a control in Lohsiriwat *et al*. study was paraffin gauze dressing that had characteristics and properties such as mesh dressing and low absorption capacity close to Bactigras® which was the control in this study. Therefore, Lohsiriwat *et al*. study was used for sample size calculation in this study.

Calculation:

Confidence level	=	95% ($\alpha = 0.05$)		$Z_\alpha = 1.96$
Power of the study	=	90% ($\beta = 0.1$)		$Z_\beta = 1.28$
Pooled variance, S_p^2	=	$\frac{S_1^2 + S_2^2}{2}$	$= \frac{2.47^2 + 3.52^2}{2}$	$= 9.2456$

Sample size: $N = \frac{(1.96 + 1.28)^2 \times 9.2456}{2^2}$

$= 24.26 \approx 25$ samples

20% drop out calculation

$N = \frac{25}{1 - 0.2}$

$= 31.25 \approx 32$ samples

Accordingly, 32 STSG donor site wounds were included in this study.

3.2.4 Inclusion criteria and exclusion criteria

Phase I clinical study

Inclusion criteria

1. Healthy volunteers
2. Age 18–65 years
3. Signed consent form

Exclusion criteria

1. Known allergy or hypersensitivity reaction to silk sericin, PHMB, or chlorhexidine acetate
2. Known skin diseases
3. Known immunocompromised diseases
4. Using antihistamine and anti-inflammatory medications or any patches within 2 weeks before and during evaluation
5. Not following all procedures

Phase II clinical study

Inclusion criteria

1. Patients who have STSG donor site wounds on the thigh
2. Aged more than 18 years
3. Signed consent form

Exclusion criteria

1. Systemic infection

2. Known allergy or hypersensitivity reaction to silk sericin, PHMB, or chlorhexidine acetate
3. Known skin diseases
4. Known immunocompromised diseases
5. Known mental defect or schizophrenia
6. Pregnancy or lactation
7. Not following all procedures

3.3 Random allocation

Phase I clinical study

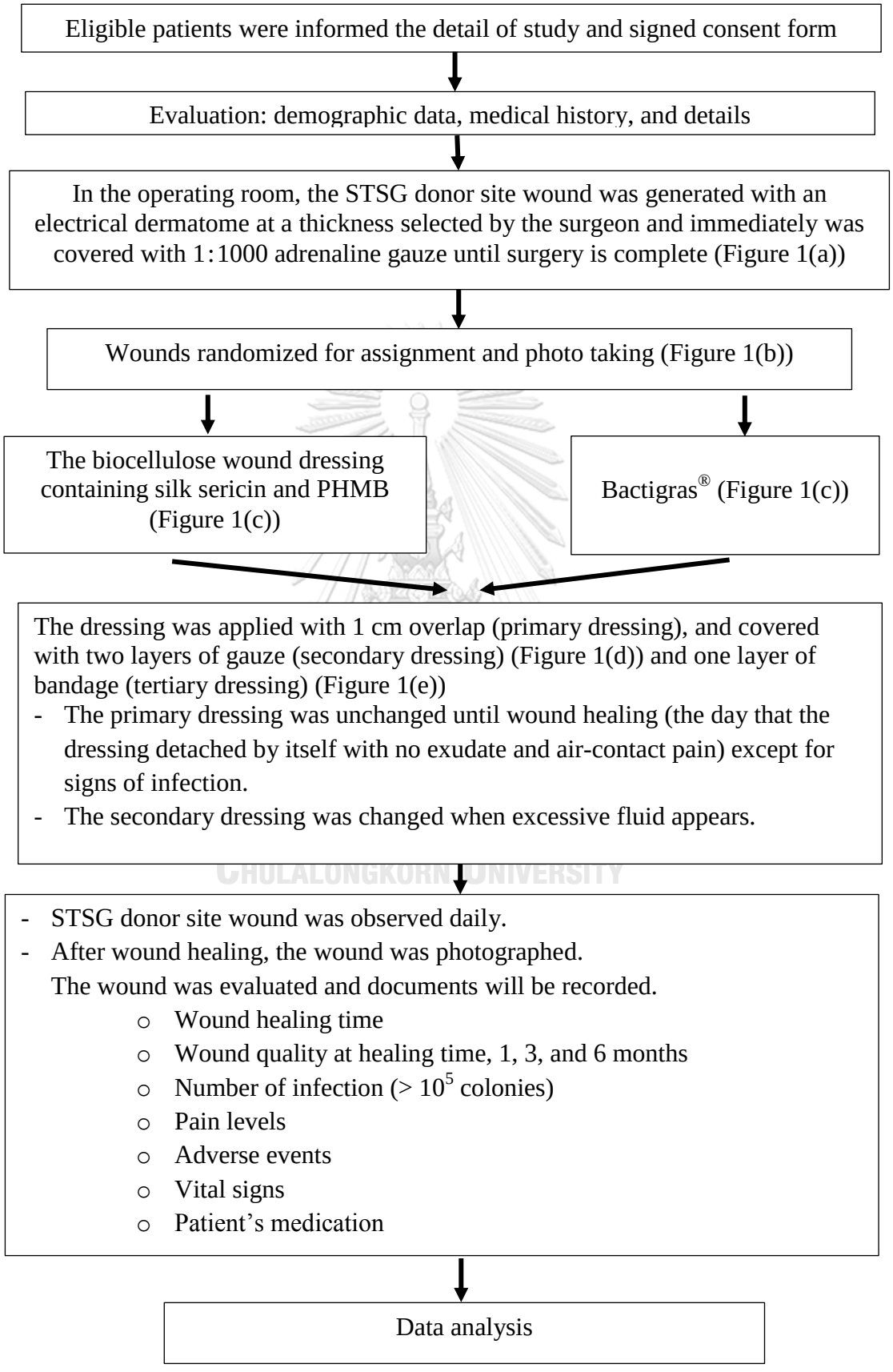
Block randomization (block size 4) was used to separate half of the back to A and half to B by using a random number table; odds equaled A and evens equaled B. A represented the biocellulose wound dressing containing silk sericin and PHMB, and B represented Bactigras®.

There were five visits in this clinical phase I study. At the first visit, the skin was randomly patched with dressings A and B (2×2 cm^2). After 3 days (the second visit), the dressings were changed to new dressings. After another 3 days (the third visit), the dressings were removed. After that, there was a free period of around 7–10 days. At the fourth visit, the skin was patched with dressings on the same area as the first visit. After 3 days, at the last visit, the dressings were removed. Erythema and melanin levels were measured by a Cutometer® in mexameter mode (Courage+Khazaka electronic GmbH, Germany) at each visit (five visits in all). Photographic assessment of edema, papules, vesicles, and bullae was performed by three dermatologists at every visit.

Phase II clinical study

Block randomization (block size 4) was used to separate half of the eligible wounds into A and half into B by using a random number table; odds equaled A and evens equaled B. A represented the biocellulose wound dressing containing silk sericin and PHMB, and B represented Bactigras®.

Flow chart of the study



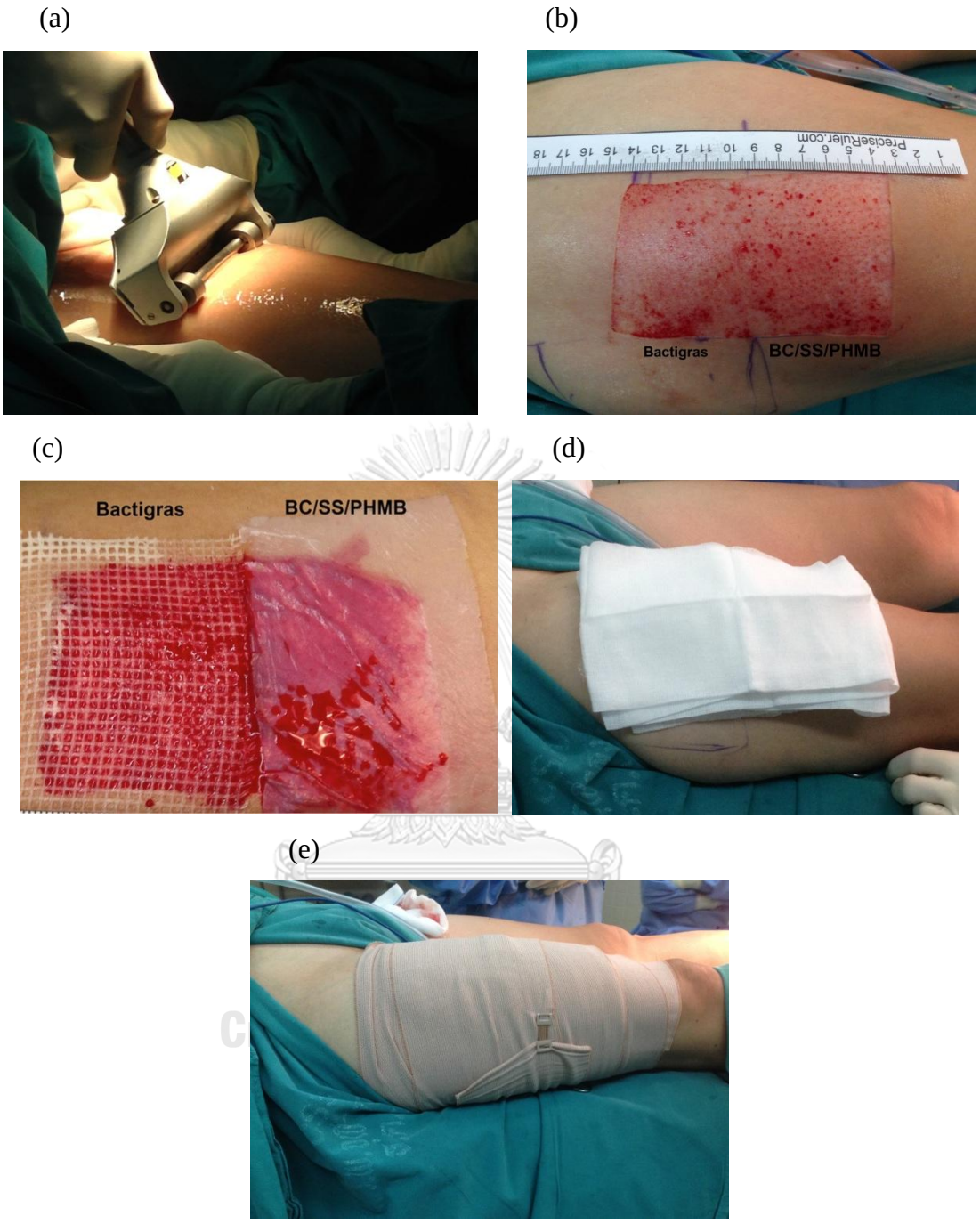


Figure 1 Wound management (a) Wound was generated with electrical dermatome at a thickness selected by the surgeon (b) Half wound randomization for assignment and photos taking (c) The biocellulose wound dressing containing silk sericin and PHMB and Bactigras® covered on the wound (d) The wound was covered with 2 layers of gauze (secondary dressing) (e) The wound was covered with 1 layer of bandage (tertiary dressing)

3.4 Wound evaluations

1. Wound healing time

   The day that the dressing detached by itself with no exudate or air-contact pain

2. Wound quality was evaluated in terms of:

   2.1 Erythema levels were the redness of the scar that referred to inflammation.

   2.2 Melanin levels were the darkness of the scar that referred to a post-inflammatory reaction (hyper or hypo pigmentation).

   2.3 Transepidermal water loss (TEWL) was a measure of the quantity of water that passed from inside a body through the epidermis to the outside environment. It referred to skin barrier function.

   - Measurement of erythema levels, melanin levels, and TEWL levels
     - Instrument:

       Cutometer® in mexameter and tewameter mode (Courage+Khazaka electronic GmbH, Germany)
     - Location and frequency:

       Four points for each wound, five times for each point (Figure 2) at healing time, 1, 3, and 6 months
     - Assessment by: A researcher

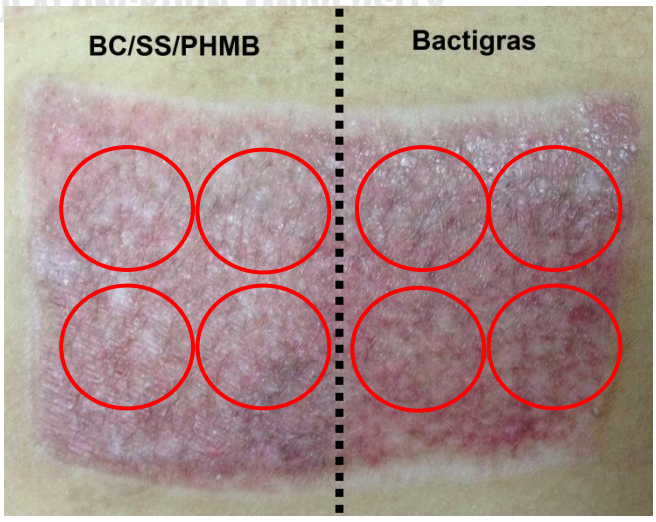


Figure 2 Wound location of measurement (red circles)

2.4 Vancouver scar scale (VSS) was used to assess the scar with four parameters: Vascularity (0–3), pigmentation (0–2), pliability (0–5), and height (0–3) (Appendix G). The maximum total score was 13. A higher VSS score represented poor scar quality.

- Location and frequency: All areas of wounds
- Assessment by: Two surgeons

3. Number of infection was the wound that had:
   - Signs of infection: Swelling, redness, bad odor, purulent discharge, assessed every day by a researcher and one surgeon
   - Swab evaluation when signs of infection were observed (bacteria $>10^5$ colonies)
   - Amount of WBC or neutrophil counts after treatment was higher than before treatment and normal range.
4. Pain levels was evaluated using Visual Analogue Scale (VAS; 0 (no pain) to 10 (unbearable pain)) by patient assessment.
5. Adverse events were evaluated in terms of:
   - Skin allergic reaction: Itching or rash, assessed by a researcher and one surgeon
   - Naranjo's algorithm assessed by a researcher
   - Systemic function: Hepatic and renal function (before and after treatment)

3.5 Definitions

Phase I clinical study definition

Healthy volunteer represented people who had healthy skin including no open wound, and no scar.

Phase I clinical study definition

1. STSG donor site wound was the wound that the skin was detached around 0.008-0.012 inch by dermatome surgical grafting instrument.
2. Wound healing time was the day that the dressing detached by itself with no exudate or air-contact pain.
3. Wound quality was the degree of excellent scar that was presented in terms of erythema levels, melanin levels, TEWL levels, and VSS.

4. Infection was the wound that had signs of infection, bacteria >10^5 colonies, and high WBC or neutrophil levels after treatment than before treatment and normal range.
5. Adverse events were any untoward occurrence in a patient treated with wound dressings.

3.6 Materials

3.6.1 Test dressings

The biocellulose wound dressing containing silk sericin and PHMB was composed of three major components, biocellulose, silk sericin, and PHMB.

The biocellulose dressings were produced from an *A. xylinum* strain (Kasetsart University, Bangkok, Thailand) in coconut water medium according to the method of Verschuren *et al*. with a slight modification [181]. Briefly, 1 L of coconut water was boiled and 50 g of sucrose, 5 g of ammonium phosphate ($(NH_4)_3PO_4$), and 10 mL of acetic acid (CH_3COOH) were added. The acetic acid was used for adjustment of solution pH (pH 4.5). Then, 10 mL of *A. xylinum* was added to the solution and the mixture was transferred to molds for fermentation. This mixture was incubated under sterilized static conditions at 30 °C for 10–12 days to form biocellulose. After that, 2% w/v aqueous sodium hydroxide (NaOH) solution was used for biocellulose washing at 70 °C until a neutral pH was attained. All processes were controlled and produced by Research X Co., Ltd. (Bangkok, Thailand).

The biocellulose dressing (10 × 10 × 0.01 cm^3) was loaded with 4 mL of 1% w/v silk sericin. After that, the dressing was loaded with 10 mL of 0.3% w/v PHMB Finally, the biocellulose wound dressing containing silk sericin and PHMB was soaked in 6 mL of 60% w/v glycerin. All processes were carried out in sterile conditions. After preparation, the dressings were packed and sterilized with gamma radiation at 25 kGy by the Thailand Institute of Nuclear Technology (Public Organization), Irradiation Certificate No 61/58 (Nakornnayok, Thailand). For the quality control, in each production, the biocellulose wound dressing containing silk sericin and PHMB were randomly tested about the released concentration. The concentration of silk sericin and PHMB that released from the dressing after 30 minutes were around 40 mcg/ml of silk sericin and 0.01%PHMB.

From previous study, Hasatsri *et al.* found that the bilayered wound dressing prepared from 1% sericin solution could accelerate wound healing [34] and promote collagen type I [182]. *In vitro* and *in vivo* study, the results showed that the biocellulose wound dressing containing silk sericin (1%w/v) could release sericin more than 40 mcg/ml resulting in collagen type I promotion [177]. Moreover, the loading concentrations of PHMB were also evaluated at 0.0125% - 0.6% in order to find concentration that had antimicrobial activity against Gram-positive and Gram-negative bacteria [177]. The result showed that the appropriated loading concentration of PHMB was at least 0.3% [177] because this dressing could release PHMB more than 0.01% that was higher than minimum bactericidal concentration of Gram-positive and Gram-negative bacteria. Therefore, in this study, the biocellulose wound dressing containing silk sericin and PHMB was loaded with 1%w/v sericin solution and 0.3%PHMB.

3.6.2 Control dressings

Bactigras® (Smith & Nephew Co., Ltd, London, UK)

3.6.3 Chemical agents

Analytical grade acetic acid (CH_3COOH), ammonium phosphate ($(NH_4)_3PO_4$), sodium hydroxide (NaOH), and other chemicals were purchased from Sigma-Aldrich, USA. Silk sericin solution was prepared from *Bombyx mori* cocoons supplied by Chul Thai Silk Co., Ltd. (Petchaboon, Thailand) using a high temperature and high pressure degumming method [147]. PHMB was kindly provided by Lonza Group Ltd. (Basel, Switzerland). Glycerin was analytical grade, obtained from Ajax Finechem (Australia).

3.7 Data collection instruments

Phase I clinical study

1. Demographic data (Appendix A)
2. Adverse event report (Appendix B)
3. Adverse event by erythema and melanin report (Appendix C)

Phase II clinical study

1. Demographic data (Appendix D)

2. Follow-up report (Appendix E)
3. Adverse event report (Appendix F)
4. VSS (Appendix G)

3.7 Data analysis and statistical evaluations

All statistical evaluations were performed using SPSS version 17.0 (SPSS. Co., Ltd., Bangkok, Thailand). Statistically significant differences of data were considered at $p < 0.05$.

Phase I clinical study

1. Demographic data were presented in terms of frequency, percentage, mean and standard deviation.
2. Comparison of erythema levels and melanin levels between two areas used a repeated measure ANOVA test.
3. Comparison of the number of infection, and amount of adverse events between two areas used chi-square tests.

Phase II clinical study

1. Demographic data were presented in terms of frequency, percentage, mean and standard deviation.
2. Comparison of wound healing time (days) used Paired t-test.
3. Comparison of erythema levels, melanin levels, TEWL levels, pain score, and VSS between two areas used Friedman test and Wilcoxon signed rank tests.
4. Comparison of the amount of infection and number of adverse events between two areas used Chi-square test.

3.8 Ethical considerations

This study was to investigate the safety of a biocellulose wound dressing containing silk sericin and PHMB in healthy volunteers, and the clinical efficacy and safety of the dressing for STSG donor site wound treatment compared with Bactigras® which was the standard dressing at King Chulalongkorn Memorial Hospital. The researcher provided all information of the study including the research objectives, expected duration, methods, benefits, and adverse events to the subjects. All subjects had to sign a consent form before starting. They could withdraw from the study at any

time, which would not impact on their regular treatment. The study was approved by the Ethic Review Committee for Research Involving Human Research Subjects, Health Sciences Group, Chulalongkorn University (COA No. 127/2557) (Appendix J) on 4th September 2014 and King Chulalongkorn Memorial Hospital Human Subjects Institutional Review Board (COA 843/2015) (Appendix J) on 3rd December 2015. The data from this study was retained as confidential and only complete results were presented.

CHAPTER 4 RESULTS

The biocellulose wound dressing containing silk sericin and PHMB was developed for wound healing (Figure 3(a)) comparing with Bactigras® (Figure 3(b)) in this study.

(a) (b)

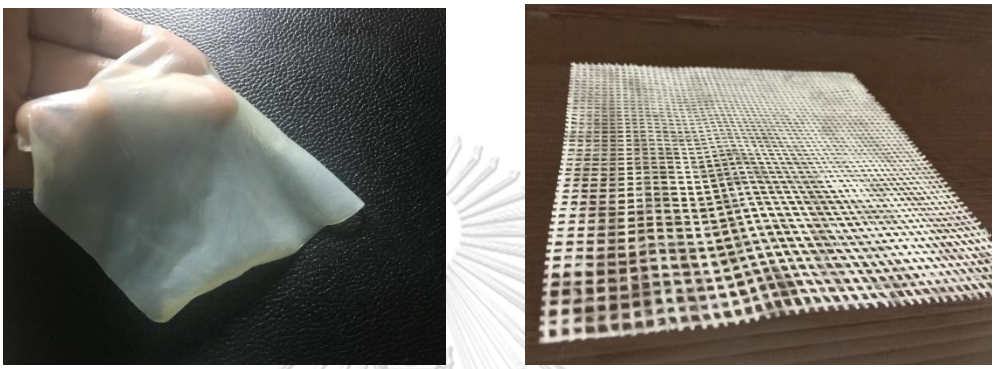


Figure 3 Dressings in this study (a) The biocellulose wound dressing containing silk sericin and PHMB (b) Bactigras®

4.1 Phase I clinical study

There were 112 healthy volunteers in this study but three volunteers were excluded because their dressing peeled off before appointment and four volunteers were excluded because they took antihistamine medicines for a common cold. Therefore, there were 105 healthy volunteers in the final results. Most were female with a normal body mass index, non-alcohol drinkers and non-smokers. Demographic data are shown in Table 17.

Table 17 Demographic data of healthy volunteers

Document		Volunteer	%
Subjects		105	100.00
Sex	Male	22	21.00
	Female	83	79.00
Average age(years ± SD (Range))		26.60 ± 10.83 (18, 60)	
Average body mass index (kg/m^2 ± SD)		21.18 ± 3.12	
Occupation	Government officer	3	2.86
	State enterprise officer	2	1.90
	Employee	31	29.51
	Students	69	65.71
Underlying disease	No	102	97.14
	Hypertension	2	1.90
	Diabetes mellitus	1	0.95
Current alcohol drinking		5	4.76
Current smoking		1	0.95

The safety of the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras® was evaluated in terms of skin erythema and melanin levels, represented by skin redness and darkness. The results showed that the levels of neither were statistically significantly different for skin covered with the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras® (Figures 4 and 5). Therefore, inflammation or post-inflammatory reaction of the skin that covered with biocellulose wound dressing containing silk sericin and PHMB was not different comparing the skin that covered with Bactigras®. Photographic assessment of edema, papules, vesicles, and bullae was performed by three dermatologists at every visit (five visits in all) (Appendix K). No significant signs of inflammation or skin irritation were found in either group (Figure 6). Edema and papules were not presented in more than 97% of subjects in both groups. Vesicles and bullae were not observed (Table 18). The results were not statistically

significantly different between the biocellulose wound dressing containing silk sericin and PHMB and Bactigras®.

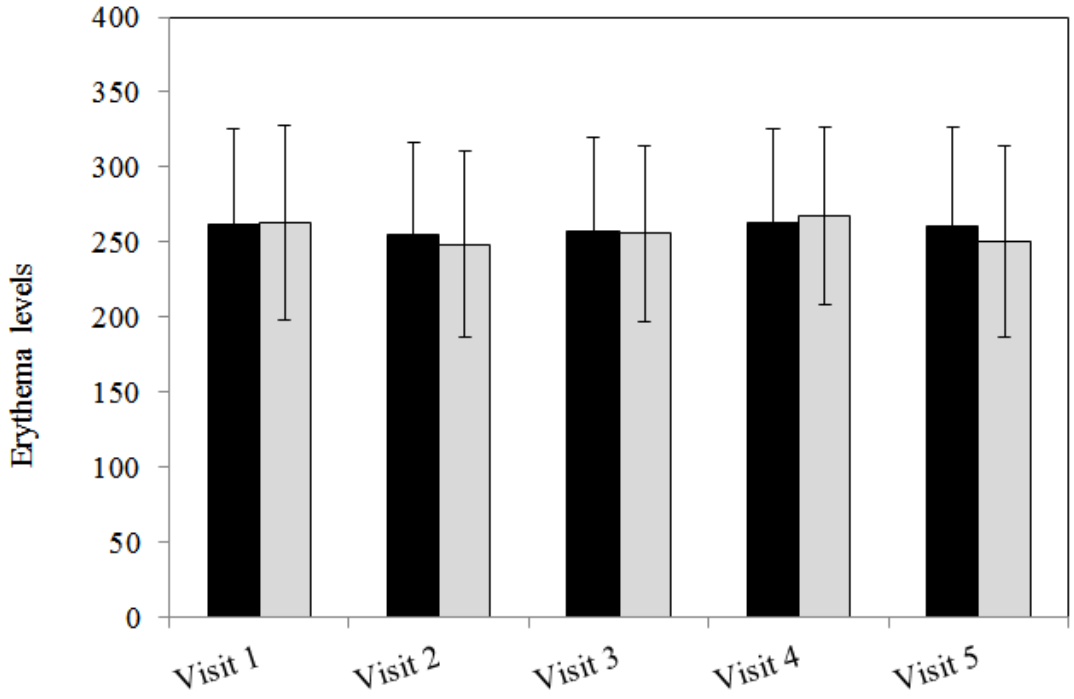


Figure 4 Erythema levels (mean ±SD) of health volunteers' skin covered with biocellulose wound dressing containing silk sericin and PHMB (black bars) and Bactigras® (grey bars; control group) at visit 1-5

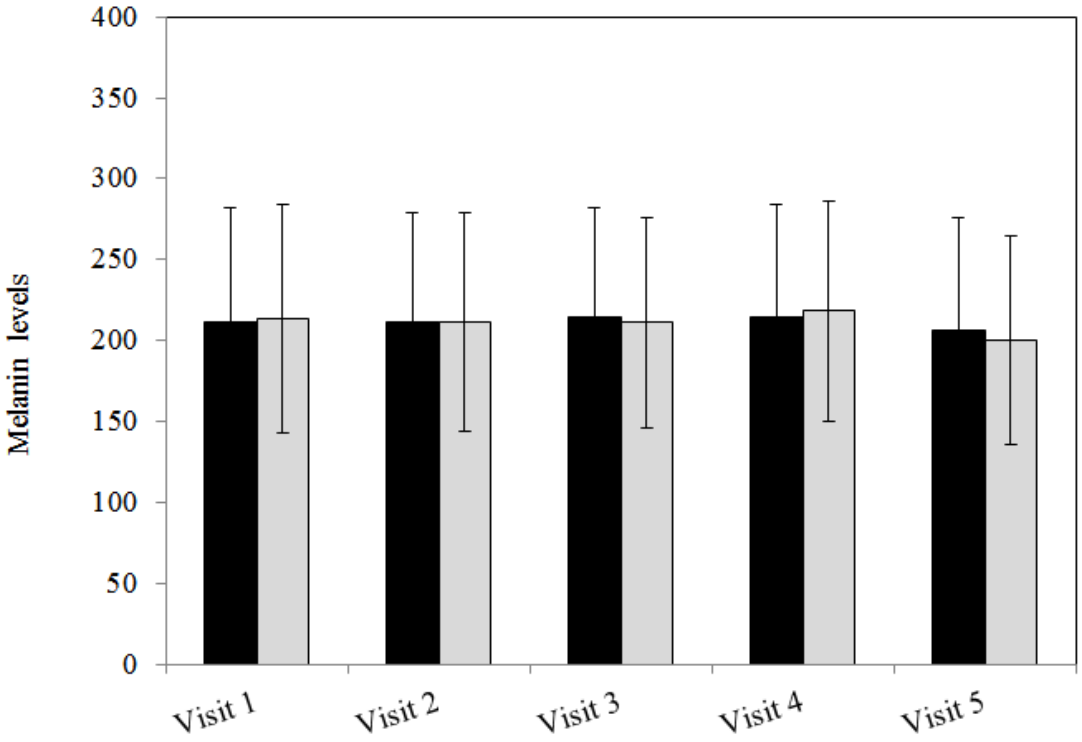


Figure 5 Melanin levels (mean ±SD) of health volunteers' skin covered with biocellulose wound dressing containing silk sericin and PHMB (black bars) and Bactigras® (grey bars; control group) at visit 1-5

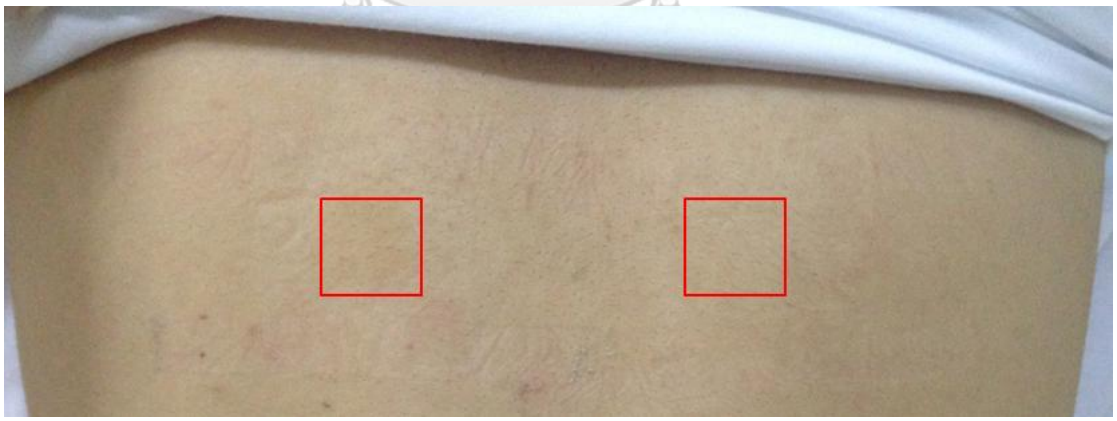


Figure 6 The skins of healthy volunteer after cover with the biocellulose wound dressing containing silk sericin and PHMB (left side) and Bactigras® (right side)

Table 18 Adverse events of biocellulose wound dressing containing silk sericin and PHMB and Bactigras® by dermatologists' assessment

Parameters		Biocellulose wound dressing containing silk sericin and PHMB (% Total subject)	Bactigras® (%Total subject)
Erythema*	Mild	39.05	50.48
	Moderate	1.0	1.0
	Severe	0.0	0.0
Edema*	Mild	1.9	0.0
	Moderate	0.0	0.0
	Severe	0.0	0.0
Papule		2.9	2.9
Vesicle		0.0	0.0
Bullae		0.0	0.0

*Erythema scale and edema scale see Appendix B

4.2 Phase II clinical study

The clinical efficacy of the biocellulose wound dressing containing silk sericin and PHMB for STSG donor sites was evaluated in terms of wound healing time, wound quality, rate of infection, pain, adverse events, and cost compared with the standard treatment (Bactigras®). Thirty-two STSG donor site wounds were enrolled in this study. Demographic data are presented in Table 19. Causes of STSG surgery were malignancy (62.5%), defected wound (31.25%), and scar contracture (6.25%). Each STSG donor site wound was divided into two parts that were randomized to be covered with the biocellulose wound dressing containing silk sericin and PHMB or Bactigras® (Figure 2).

Table 19 Demographic data of STSG donor site patients

Documents	Subjects	Percentage
Subjects	21	100.00
Sex		
Male	16	76.19
Female	5	23.81
Age (years) (mean ± SD (Range))	60.00 ± 19.24 (25,90)	
Body mass index (kg/m^2) (mean ± SD (Range))	23.79 ± 5.77 (16.44,42.24)	
Comorbidity	14	66.67
Dyslipidemia	9	42.86
Hypertension	11	52.38
Cardiovascular disease	3	14.29
Diabetes mellitus	5	23.81
Renal Insufficiency	1	4.76
Smoking	6	28.57
Alcohol drinking	2	9.52
Allergic history	3	14.29
Ibuprofen	1	4.76
Morphine	1	4.76
Sulfonamide	1	4.76

Documents	Subjects	Percentage
Wounds	32	100.00
Sex		
Male	27	84.38
Female	5	15.63
Area		
Right thigh	20	62.50
Anterior	13	40.62
Posterior	0	0.00
Lateral	7	21.88
Left thigh	12	37.50
Anterior	4	12.50
Posterior	1	3.13
Lateral	7	21.88
Wound area (cm2) (mean ± SD (Range))	60.89 ± 19.82 (28.70,104.71)	
STSG thickness (inch) (mean ± SD (Range))	0.01 ± 0.0014 (0.008,0.012)	

4.2.1 Wound healing time of STSG donor sites

An STSG donor site wound was a wound that involves the epidermis and some part of the dermis because of skin detachment by instrument. The standard treatment for STSG donor site wounds in King Chulalongkorn Memorial Hospital was Bactigras®. In general, the wound healing time of this treatment was the day that the dressing detached by itself with no exudate and no air-contact pain.

The results of this study demonstrated that the wound healing time of both dressings was around 19 ± 5 days. There was no statistically significant difference between groups (Figures 7 and 8) (Appendix L).

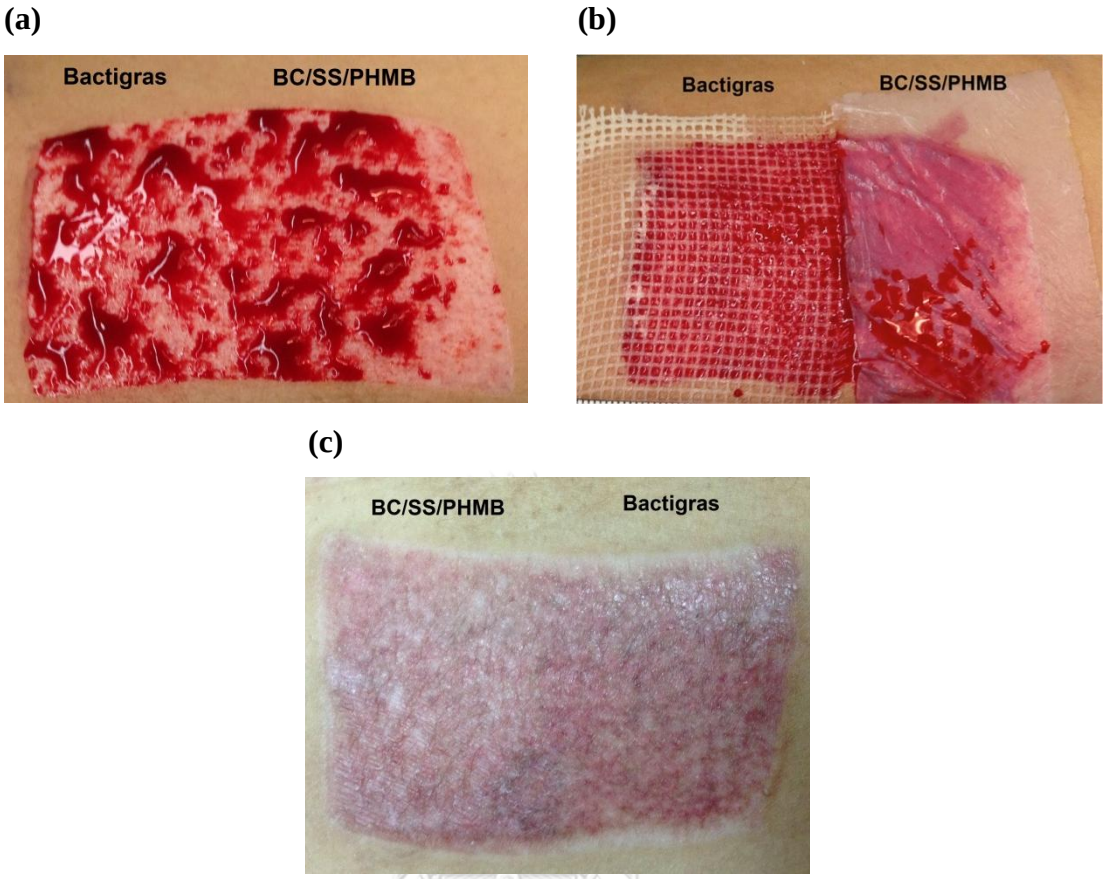


Figure 7 Wound appearance (a) Split-thickness skin graft donor site wound at day 0, (b) Split-thickness skin graft donor site wound covered with biocellulose wound dressing containing silk sericin and PHMB (BC/SS/PHMB) (Right side) and Bactigras® (Left side), (c) Split-thickness skin graft donor site wound at healing day

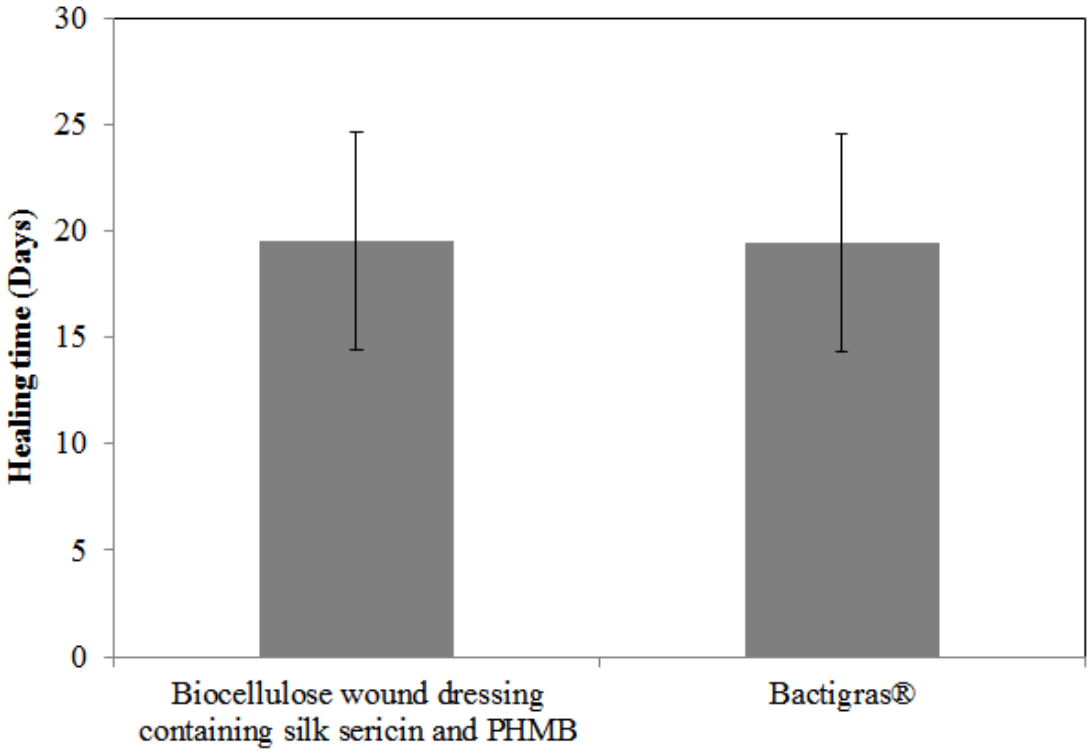


Figure 8 Wound healing time (mean ± SD) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB and Bactigras® (p = 0.161)

4.2.2 Wound quality of STSG donor sites

Wounds treated with good properties' dressings led to better wound quality. The wound quality of STSG donor sites was presented in terms of melanin levels, erythema levels, TEWL levels, and VSS.

4.2.2.1 Melanin levels

A post-inflammatory reaction occurred after skin inflammation or injury. A change in skin color was the most common effect. Melanocytes were stimulated by cytokines, chemokines, and inflammatory mediators, resulting in overproduction (hyperpigmentation) or lower production (hypopigmentation) of melanin and irregular pigment dispersion [183]. The melanin levels represented the darkness of the scar and referred to post-inflammatory reaction. A good quality wound should have melanin close to those of normal skin. They were measured by using a Cutometer® in mexameter mode (Courage+Khazaka electronic GmbH,

Germany) at four points for each wound, five times for each point (Figure 2) at healing time, 1, 3, and 6 months. The results were compared with normal skin. If the results were higher than for normal skin, they were interpreted as hyperpigmentation. If the results were lower than for normal skin, they were interpreted as hypopigmentation.

Melanin levels of wounds treated with both dressings are shown in Figure 9. Melanin levels of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras® group at healing time and 1 month were significantly lower than normal skin. However, there were no significant differences at 3 and 6 months.

At healing time and 1 month, the melanin levels of the biocellulose wound dressing containing silk sericin and PHMB-treated group were significantly lower than the Bactigras® group (160.33 ± 134 vs 251.70 ± 159; p = 0.000014 and 291.10 ± 174 vs 295.08 ± 212; p = 0.00033, respectively). Therefore, post-inflammatory reaction of biocellulose wound dressing containing silk sericin and PHMB-treated group was lower than the Bactigras® group. However, 3 and 6 months after wound healing, there was no significant difference between groups.

The melanin levels of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras® group continuously significantly increased until 3 months.

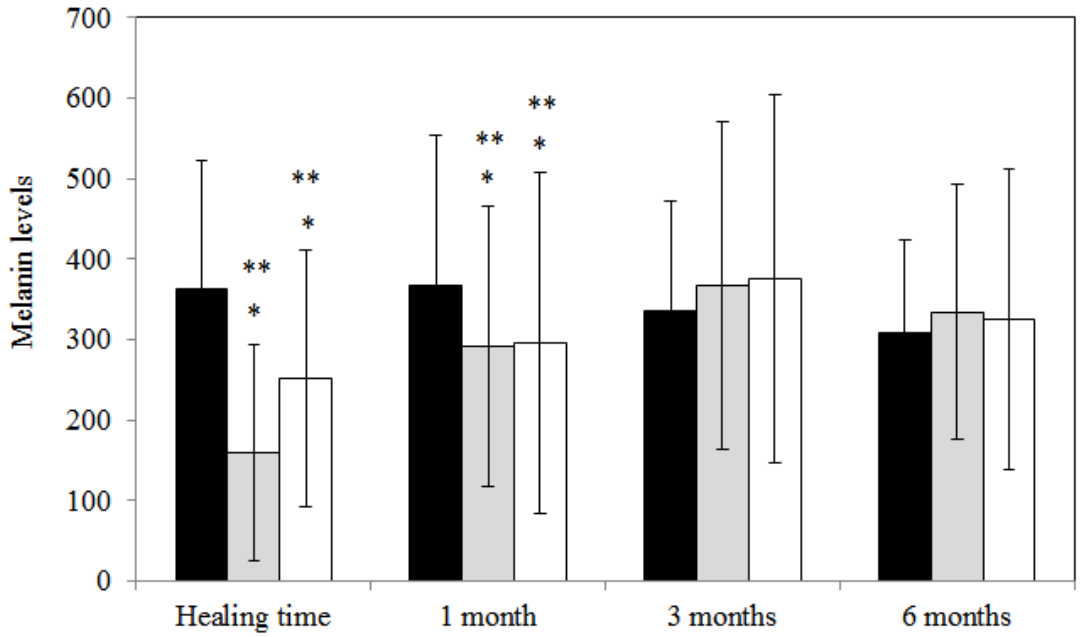


Figure 9 Melanin levels (median ±IQR) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB (grey bars) and Bactigras® (white bars; control group) at healing time, 1, 3, and 6 months after wound healing comparing with normal skin (black bar) (*Significant difference when compared with the value of normal skin (p = 7x10^{-7}, p = 7x10^{-7}, p = 0.00001, p = 0.015, respectively), **Significant difference when compared with the value of Bactigras® (p = 0.000014, p = 0.00033, respectively)

4.2.2.2 Erythema levels

Inflammation occurred after skin injury. The erythema levels represented the redness of the scar and referred to inflammation. A good quality wound should have erythema levels close to those of normal skin. They were measured by using a Cutometer® in mexameter mode (Courage+Khazaka electronic GmbH, Germany) at four points for each wound, five times for each point (Figure 2) at healing time, 1, 3, and 6 months. If the erythema levels were higher than normal skin, it was interpreted as inflammation.

Figure 10 shows the erythema levels of the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras®-treated group. The erythema levels of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the

Bactigras® group were significantly higher than normal skin at all-time points.

The erythema levels of the biocellulose wound dressing containing silk sericin and PHMB-treated group were significantly lower than the Bactigras® group (508.75 ± 138.39 vs 571.20 ± 133.16; $p = 0.013$ and 465.53 ± 97.46 vs 512.03 ± 149.56; $p = 0.00033$, respectively) at healing time and 1 month after wound healing. Therefore, inflammation of biocellulose wound dressing containing silk sericin and PHMB-treated group was lower than the Bactigras® group. However, 3 and 6 months after wound healing, there were no significant differences between groups.

The erythema levels of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras® group continuously significantly decreased until 6 months.

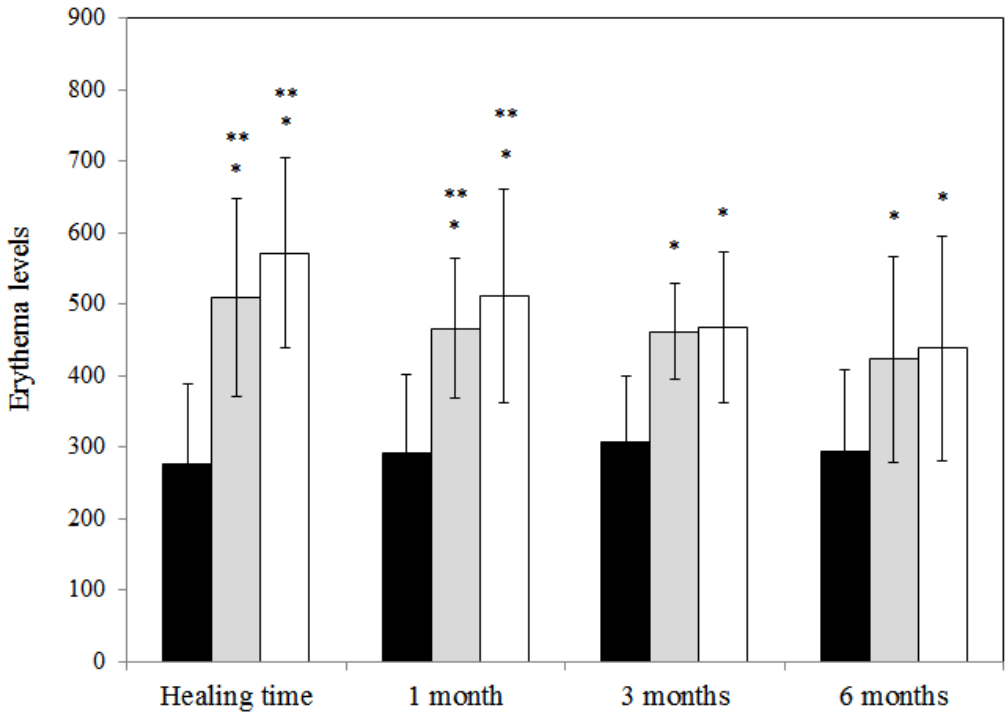


Figure 10 Erythema levels (median ±IQR) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB (grey bars) and Bactigras® (white bars; control group) at healing time, 1, 3, and 6 months after wound healing comparing with normal skin (black bar) (*Significant difference when compared with the value of normal skin ($p = 9x10^{-7}$, $p = 7x10^{-7}$, $p = 1x10^{-6}$, $p = 8x10^{-7}$, $p =$

$8x10^{-7}$, $p = 8x10^{-7}$, $p = 2x10^{-6}$, $p = 4x10^{-6}$, respectively), **Significant difference when compared with the value of Bactigras® ($p = 0.013$, $p = 0.00033$, respectively)

4.2.2.3 Transepidermal water loss levels (TEWL)

TEWL was a measure of the quantity of water that passes from inside a body through the epidermis to the outside environment. It referred to skin barrier function. After wound healing, the expected scar should have a low TEWL levels or one close to that of normal skin. It was measured by using a Cutometer® in tewameter mode (Courage+Khazaka electronic GmbH, Germany) at four points for each wound, five times for each point (Figure 2) at healing time, 1, 3, and 6 months. The results were compared with normal skin. If the results were higher than for normal skin, they were interpreted as poor skin barrier function.

The TEWL levels of the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras®-treated group are shown in Figure 11. The TEWL levels of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras® group were significantly higher than normal skin at all-time points.

At healing time, the results showed that the TEWL levels of the biocellulose wound dressing containing silk sericin and PHMB-treated group was not significantly different to the Bactigras® group. However, the TEWL levels of the biocellulose wound dressing containing silk sericin and PHMB-treated group was significantly less than the Bactigras® group after 1, 3, and 6 months (13.08 ± 6.08 vs 16.86 ± 6.39; $p = 0.000025$, 9.67 ± 5.39 vs 10.68 ± 6.51; $p = 0.000028$, and 7.69 ± 7.16 vs 9.63 ± 7.53; $p = 5 \times 10^{-6}$, respectively). Therefore, skin barrier function of biocellulose wound dressing containing silk sericin and PHMB-treated group was better than the Bactigras® group.

The TEWL levels of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras® group continuously significantly decreased until 3 months.

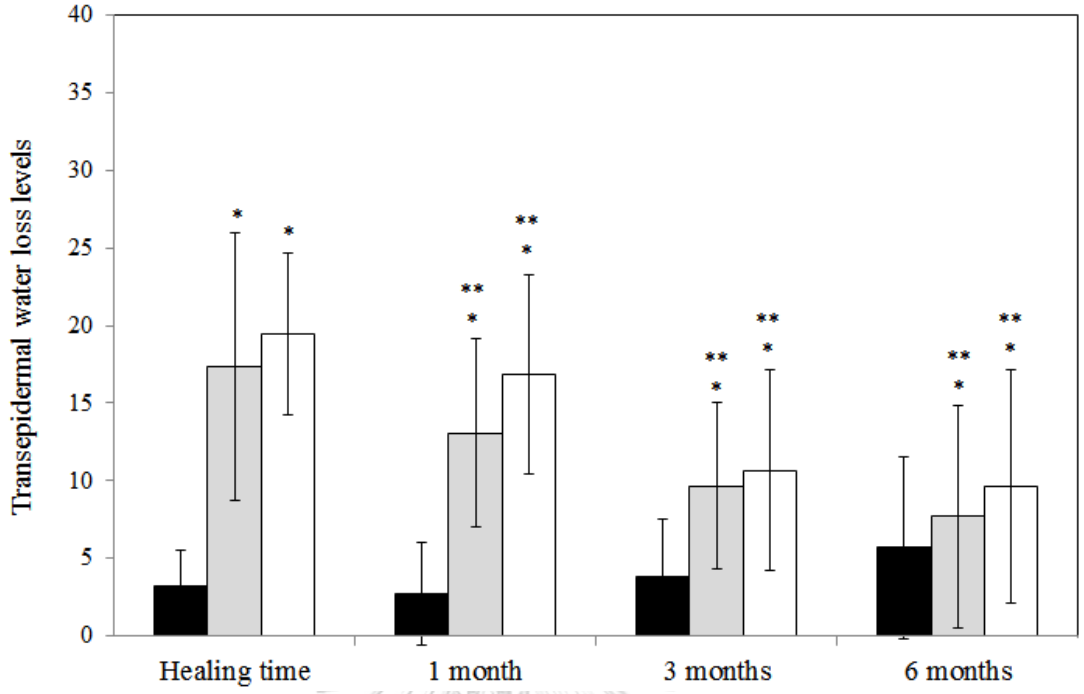


Figure 11 Transepidermal water loss levels (median ±IQR) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB (grey bars) and Bactigras® (white bars; control group) at healing time, 1, 3, and 6 months after wound healing comparing with normal skin (black bar) (*Significant difference when compared with the value of normal skin ($p = 8x10^{-7}$, $p = 8x10^{-7}$, $p = 8x10^{-7}$, $p = 8x10^{-7}$, $p = 9x10^{-7}$, $p = 8x10^{-7}$, $p = 0.000086$, $p = 0.000017$, respectively), **Significant difference when compared with the value of Bactigras® ($p = 0.000025$, $p = 0.000028$, $p = 5x10^{-6}$, respectively)

4.2.2.4 Vancouver scar scale (VSS)

VSS was used to assess the scar with four parameters that were vascularity (0–3), pigmentation (0–2), pliability (0–5), and height (0–3) (Appendix G). The maximum total score was 13. A higher VSS score represented poor scar quality. They were measured at all areas of wounds by two surgeons. VSS total scores were compared between sample and control. If the total score of the sample was lower than the control, it was interpreted that the scar quality of the sample was better than the control.

VSS was used to assess the scar with four parameters that were vascularity, pigmentation, pliability, and height.

The vascularity scores of the dressings were not significantly different. They continuously decreased over time (Figure 12).

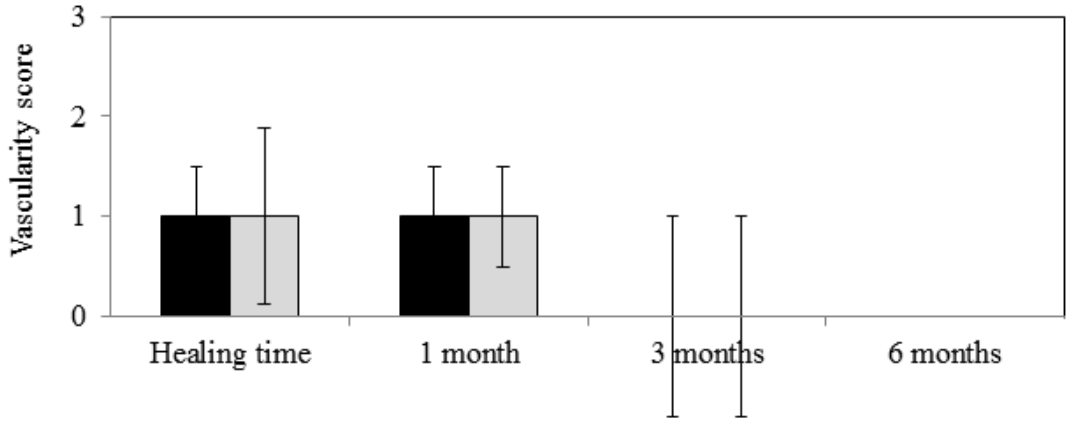


Figure 12 Vascularity score (median ±IQR) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB (black bars) and Bactigras® (grey bars; control group) at healing time, 1, 3, and 6 months after wound healing

The pigmentation scores of both dressings continuously increased over time (Figure 13). However, the pigmentation score of the biocellulose wound dressing containing silk sericin and PHMB-treated group was lower than the Bactigras® group 1 month after wound healing (0.5 ± 1 vs 0.75 ± 1; $p = 0.013$).

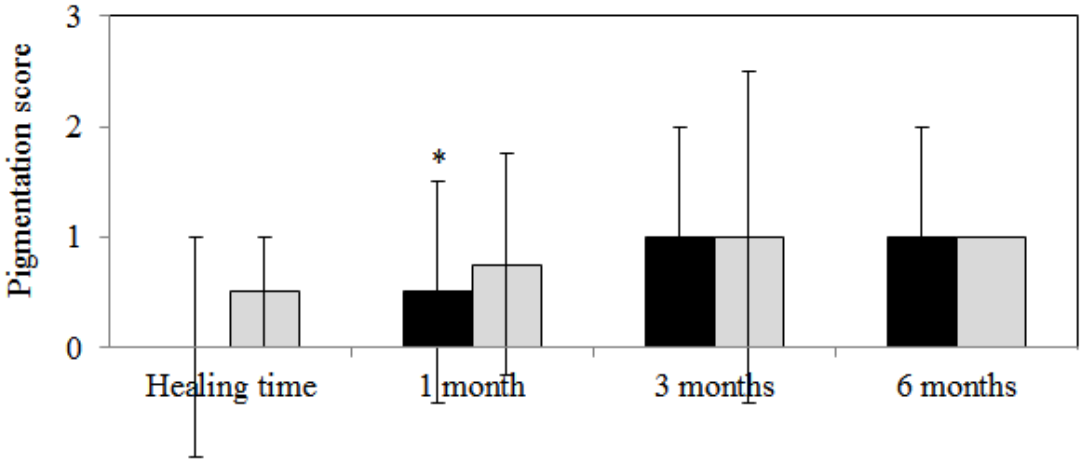


Figure 13 Pigmentation score (median ±IQR) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB (black bars) and Bactigras® (grey bars; control group) at healing time, 1, 3, and 6 months after wound healing ($p = 0.013$)

The pliability scores of the dressings were not significantly different. They continuously decreased over time (Figure 14).

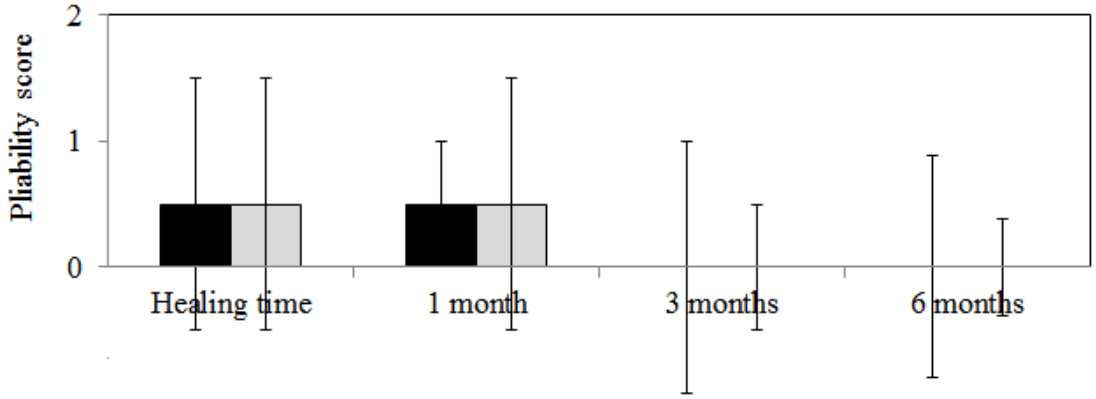


Figure 14 Pliability score (median ±IQR) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB (black bars) and Bactigras® (grey bars; control group) at healing time, 1, 3, and 6 months after wound healing

The height scores of the dressings were not significantly different. They continuously decreased over time (Figure 15).

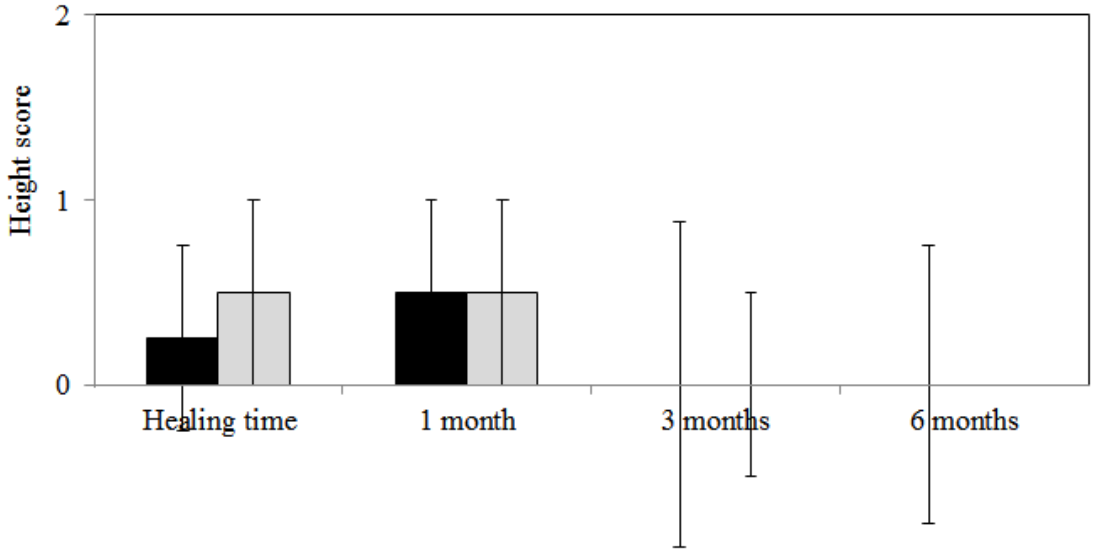


Figure 15 Height score (median ±IQR) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB (black bars) and Bactigras® (grey bars; control group) at healing time, 1, 3, and 6 months after wound healing

The results demonstrated that the overall VSS scores of the dressings were not significantly different. However, the VSS score of the

biocellulose wound dressing containing silk sericin and PHMB-treated group was lower than the Bactigras® group at healing time and 1 month after wound healing (2 ± 1.5 vs 2.5 ± 1.5; p = 0.274 and 2 ± 1.5 vs 2.5 ± 1.5; p = 0.069, respectively) because the pigmentation score of the biocellulose wound dressing containing silk sericin and PHMB-treated group was lower than the Bactigras® group (0.0 ± 1 vs 0.5 ± 0.5; p = 0.564 and 0.5 ± 1 vs 0.75 ± 1; p = 0.013, respectively). Moreover, the VSS score of both groups continuously decreased at 3 and 6 months (Figure 16).

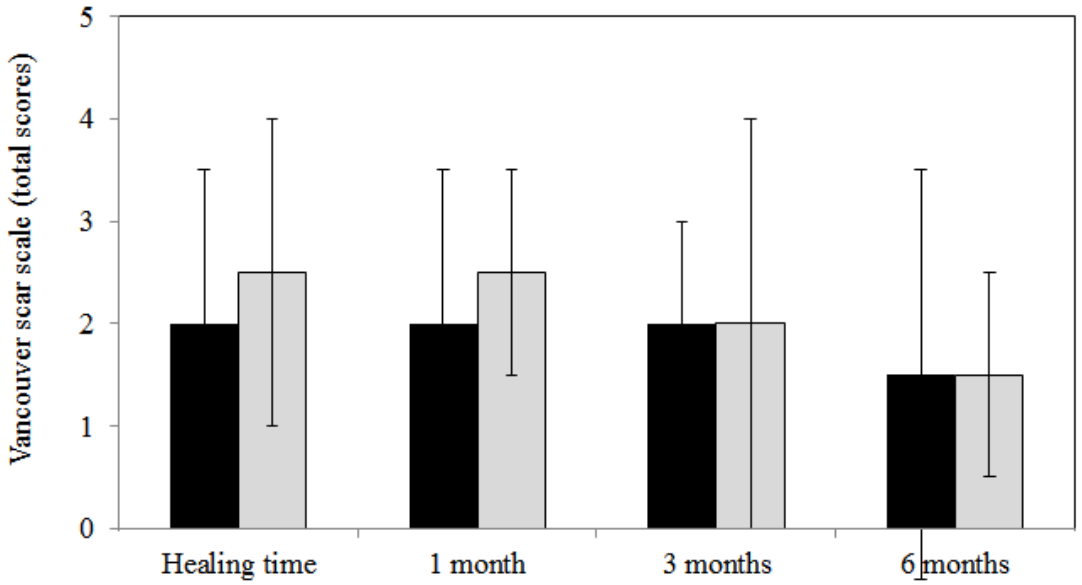


Figure 16 Vancouver scar scale (median ±IQR) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB (black bars) and Bactigras® (grey bars; control group) at healing time, 1, 3, and 6 months after wound healing

4.2.3 Number of STSG donor sites infection

No signs of infection (swelling, redness, bad odor, or purulent discharge) were observed in wounds that were covered with either dressing. All median patient temperatures (day 1–5) were lower than 37 °C. Biochemistry tests including white blood cell count and neutrophils presented in the normal range at days 0 and 5 after operation (Table 19). After treatment, there was one patient who had a higher WBC than normal. There were two patients who had a higher

neutrophil level than normal level. However, both levels were lower than before treatment (Appendix I).

4.2.4 Pain levels of STSG donor sites

Pain scores of wounds covered with the biocellulose wound dressing containing silk sericin and PHMB and Bactigras® gradually decreased from days 1–5. However, the pain scores of wounds covered with the biocellulose wound dressing containing silk sericin and PHMB were statistically significantly lower than wounds covered with Bactigras® at all-time points (4 ± 2.75 vs 7 ± 2.75; $p = 0.0000092$, 3.5 ± 2 vs 7 ± 3; $p = 0.000014$, 1 ± 1 vs 4 ± 2; $p = 3 \times 10^{-6}$, 0 ± 1 vs 3 ± 1; $p = 4 \times 10^{-6}$, and 0 ± 1 vs 3 ± 1; $p = 4 \times 10^{-6}$, respectively). The pain scores of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras® group continuously significantly decreased until day 5 (Figure 17).

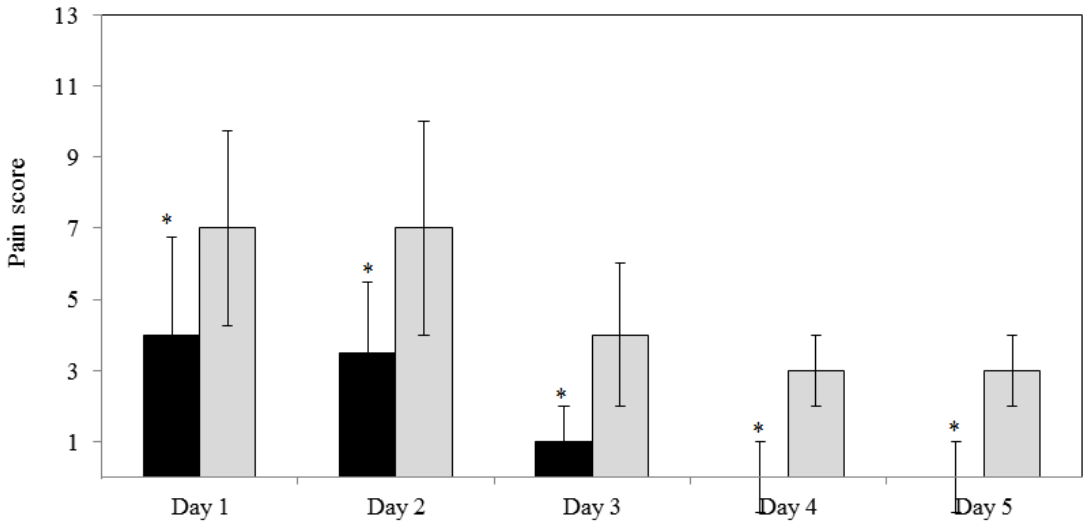


Figure 17 Pain score (median ±IQR) of STSG donor sites wound treated with biocellulose wound dressing containing silk sericin and PHMB (black bars) and Bactigras® (grey bars; control group) at day 1-5 after wound healing (*Significant difference when compared with the value of control group ($p = 0.0000092$, $p = 0.000014$, $p = 3\text{x}10^{-6}$, $p = 4\text{x}10^{-6}$, $p = 4\text{x}10^{-6}$, respectively)

4.2.5 Adverse events

There were no adverse skin reactions including rash, itching, edema, or papules. Systemic reactions including renal function and hepatic function also showed normal levels at days 0 and 5 after operation (Table 19).

Table 20 Biochemistry test (infection, renal function and hepatic function) of STSG donor site patients at day 0 and day 5 after operation

Biochemistry[a]	Normal value	Day 0 Median ± IQR	Day 5 Median ± IQR
WBC ($x10^3$/ul)	4.50 - 11.00	10.80 ± 4.07	7.72 ± 3.20
Neutrophil ($x10^3$/ul)	1.80 - 7.80	7.50 ± 5.16	5.70 ± 2.73
BUN (mg/dl)	7.00 – 20.00	14.00 ± 8.00	14.00 ± 7.50
Cr (mg/dl)	0.50 – 1.00	0.94 ± 0.34	0.77 ± 0.36
AST (U/L)	0.00 – 35.00	26.00 ± 11.00	28.00 ± 21.00
ALT (U/L)	0.00 – 40.00	18.00 ± 22.50	21.00 ± 15.00
ALP(U/L)	40.00 – 120.00	64.00 ± 34.00	84.00 ± 31.00
Total bilirubin	0.20 – 1.20	0.56 ± 0.34	0.45 ± 0.38

[a] WBC: White blood cells, BUN: Blood urea nitrogen, Cr: Creatinine, AST: aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase

After treatment, there was one patient who had a higher BUN level than normal level. There were five patients who had a higher creatinine level than normal level. However, both levels were close to those recorded before treatment, which were also higher than normal. Furthermore, there were five patients who had a higher AST level than normal level. There were three patients who had a higher ALT level than normal level. There were two patients who had a higher ALP level than normal level and one patient who had a higher total bilirubin level than normal level. However, all levels were close to those recorded before treatment, which were also higher than normal (Appendix I).

4.2.6 Cost of dressings

The cost of the two dressings was evaluated per square centimeter. The cost of Bactigras® (overall' cost) was higher than the biocellulose wound dressing containing silk sericin and PHMB (materials' cost) (0.17 vs 0.07 baht).

CHAPTER 5 DISCUSSION AND CONCLUSIONS

Discussion

An STSG donor site wound is a wound that has loss of the epidermis and some part of the dermis. It usually produces exudate and may be infectious, leading to delayed wound healing. The concern of most patients is pain leading to a decrease in quality of life. Therefore, developing a dressing with wound healing acceleration, good scar quality production, pain reduction, infection protection, few adverse reactions, and low cost is of benefit for these wounds.

From our previous study, an *in vitro* test, we found that the biocellulose wound dressing containing silk sericin and PHMB contained appropriate amounts of silk sericin and PHMB. It could accelerate L929 cells to promote collagen type I and had antimicrobial activity against Gram-positive and Gram-negative bacteria [177]. The interaction between silk sericin and PHMB was also studied and presented in our previous research [177]. We found that silk sericin and PHMB had a weak interaction, which slightly affected the amide I region or the secondary structure of the protein, indicating a change in the secondary structure of the protein. However, this interaction did not affect the activity of silk sericin or PHMB in the dressing. Moreover, interaction between the biocellulose dressing and other molecules (silk sericin and PHMB) was not observed. The physical properties of this dressing were moderate wound absorption, no degradation of enzymes, and non-adhesion to the skin. Therefore, it could absorb exudate, did not macerate or split out, stayed on the wound, and reduced pain. In *in vivo* tests [24], the efficacy of the biocellulose wound dressing containing silk sericin and PHMB compared with Bactigras® on full-thickness skin wounds in 24 rats indicated that the area fraction of collagen, a wound healing acceleration parameter of the biocellulose dressing, was significantly higher than controls on day 14. The wound size reduction of wounds treated with the biocellulose dressing was also significantly higher than Bactigras® on day 14, and wounds were completely closed by day 21 [24]. There was one infected wound in the biocellulose dressing group and four infected wounds in the Bactigras® group [24]. The safety study of the biocellulose wound dressing containing silk sericin and PHMB compared

with Bactigras® in rats showed that the rats implanted with the biocellulose dressing were healthy and had less irritation and inflammation [24].

For the phase I clinical study in healthy volunteers, erythema and melanin levels of skin, representing skin redness and darkness, were evaluated. The results showed that the levels for skin covered with the biocellulose wound dressing containing silk sericin and PHMB were not statistically significantly different when compared with Bactigras®. Moreover, the melanin and erythema levels in this study were close to those of normal skin. These results agreed with Maenthaisong *et al.* [184], who showed that the mean melanin levels in healthy Thai females was around 230 and the erythema levels was around 220. Siriteintong *et al*. also found that the mean melanin levels in healthy Thai females was around 220 and the erythema levels was around 250 [13]. No significant signs of inflammation or skin irritation were found in either group. Edema and papules were not presented in more than 97% of subjects in both groups. Vesicles and bullae were not observed [24]. These results conformed to those of Ibarra de Palacios *et al*. They investigated the skin irritation potential of a 12.5 cm^2 estradiol transdermal patch (Climara®, USA) in 99 healthy postmenopausal women. The safety results claimed that 3.0% had moderate erythema, 1.0% severe erythema, 1.0% mild edema, and 34.3% papules [185].

It was expected that the biocellulose wound dressing containing silk sericin and PHMB would accelerate wound healing. The dressing's property of keeping the wound moist provides a suitable environment for wound healing. Voineskos *et al* carried out a systematic review of skin graft donor site dressings, comparing moist wound dressings with non-moist wound dressings [45]. They reported that moist wound dressings had a faster healing rate, less pain, and were less expensive. These results agreed with the study of Wiechula about the use of moist wound-healing dressings in the management of STSG donor sites [3]. The numbers of day taken for wound healing for hydrocolloid dressings were significantly less than other moist products. In chronic venous insufficiency and lower leg ulceration, Alvarez *et al.* reported that the healing time (> 75% re-epithelialization) of wounds treated with biocellulose was lower than that of the non-adherent petrolatum emulsion-impregnated cellulose acetate gauze (control) group. The percentage wound size reduction of wounds treated with biocellulose was higher than that of the control

group after 6 and 12 weeks [7]. Moreover, the biocellulose wound dressing containing silk sericin and PHMB contained a wound healing acceleration agent. Silk sericin from Chul Thai Silk 1/1 (0.2–1.0 mg/mL) could activate growth of L929 mouse fibroblast cells and the production of collagen type I in cell culture medium [10]. It could increase the number of skin fibroblast cells and collagen production, important factors in the wound healing process, to 250% in 72 h [149]. Furthermore, it accelerated proliferation of mammalian cells including human epithelial cells and human hepatoblastoma cells [146].

In STSG donor site wounds, Siritientong *et al*. found that the healing time of wounds treated with ethyl alcohol-precipitated silk sericin/PVA scaffolds was significantly less than that of the Bactigras® (control) group [13]. These results agreed with Hasatsri *et al*.. They found that the healing time of wounds treated with a bilayered wound dressing containing 1% silk sericin was significantly less than that of the Bactigras® (control) group [34]. The wound healing time of partial-thickness burn wounds treated with a silver zinc sulfadiazine cream containing silk sericin was also less than that of silver zinc sulfadiazine cream without silk sericin [186]. From all these studies, it indicated that sericin had significant effect on wound healing by acceleration of healing process, which might due to collagen promotion activity as previously reported [10].

In this study, the biocellulose wound dressing containing silk sericin and PHMB was evaluated in STSG donor site wounds compared with a standard dressing (Bactigras®) in King Chulalongkorn Memorial Hospital. The wound healing time in this study was the day that the dressing detached by itself with no exudate and no air-contact pain. The results showed that the wound healing time of both dressings was 19 ± 5 days and it was not statistically different between groups (Figure 6). There are many factors which affect STSG donor site wound healing including the depth, site, and size of the wound, along with the age of the patient and comorbidity. According to previous research, it was reported that elderly patients could have delayed wound healing time and a longer wound healing time, as long as 21 days [28], than young patients (7–14 days) because of deteriorated blood supply, declined immunity, and incomplete body function [4]. The patients in this study were old patients with an average age of about 61 years and the eldest was around 90 years. The results in this

study conformed to the study of Hakkarainen *et al.* [187]. They studied the efficacy of a nanofibrillar cellulose wound dressing compared with a synthetic copolymer of polylactide, trimethylene carbonate, and e-caprolactone (Suprathel®) in STSG donor site wounds. The patients in this comparison study had an average age around 56 years. The results found that the nanofibrillar cellulose wound dressing self-detached on average around 18 days after operation and Suprathel® self-detached on average around 22 days after operation [187]. Davidson *et al*. also reported that the older partial-thickness wound patient group (more than 50 years old) treated with a keratin dressing had a lower epithelialization percentage than younger patients [188]. Lauchli *et al*. studied the efficacy of calcium alginate versus polyurethane film dressings in STSG donor site treatment. The average ages of the groups were 72.1 and 78.6 years, respectively. The results demonstrated that the healing time of the dressings was not significantly different (18.8 vs 21.9 days) [189]. Moreover, this study found that some patients had a lot of wound exudate, which might prolong the wound healing time of both dressings. Excessive wound exudate also had a direct effect on wounds treated with the biocellulose wound dressing containing silk sericin and PHMB. Because the biocellulose wound dressing containing silk sericin and PHMB had a low to moderate wound exudate absorption, overcapacity of the wound dressing led to maceration and loss of the dressing's wound healing properties. Bactigras® had no wound exudate absorption property. However, in this study the wounds of both dressings were covered with gauze that could absorb the remaining exudate. Therefore, for excessively exudative wounds, the absorption properties of the two dressings were not different. These cases might be the cause of the non-significant difference of wound healing time between groups in this study. Moreover, sericin was a hydrophilic agent that was easy to dissolve. When the patients had excessively exudative wound, high amounts of sericin were dissolved in that exudate led to loss of sericin healing properties. Consequently, the biocellulose wound dressing containing silk sericin and PHMB might not be appropriate for high-exudate STSG donor site wounds. In addition, some reports found that the healing time of wounds treated with silk sericin was not significantly different to controls. The size of full-thickness wounds treated with 8% silk sericin cream was also not significantly different when compared with silver sulfadiazine cream after 15 days, but the collagen deposits of wounds treated

with 8% silk sericin cream were higher than silver sulfadiazine cream-treated wounds [190]. This result conformed to the scar quality results in this study.

A post-inflammatory reaction occurred after skin inflammation or injury. A change in skin color was the most common effect. Melanocytes were stimulated by cytokines, chemokines, and inflammatory mediators, resulting in overproduction (hyperpigmentation) or lower production (hypopigmentation) of melanin and irregular pigment dispersion [183]. Therefore, wounds treated with dressings with good properties would have higher wound quality. The wound quality of STSG donor sites was presented in terms of melanin levels, erythema levels, TEWL levels, and VSS score.

The melanin levels represented the darkness and the erythema levels represented the redness of the scar, measured by a Cutometer® in mexameter mode (Courage+Khazaka electronic GmbH, Germany). A good quality wound should have melanin and erythema levels close to those of normal skin. If the results were higher or lower than normal skin, it represented hyperpigmentation or hypopigmentation, respectively, with inflammation and poor scar quality. The scar quality of the biocellulose wound dressing containing silk sericin and PHMB-treated group seemed to be better than the Bactigras®-treated group. The melanin levels of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras® group at healing time and 1 month were significantly lower than normal skin, following the amount of melanocytes developing. The levels were close to those of normal skin at 3 and 6 months. From these results, it might be concluded that neither the biocellulose wound dressing containing silk sericin and PHMB nor Bactigras® induced hyper- or hypopigmentation. The erythema levels of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras®-treated group were significantly higher than normal skin at all-time points. This result agreed with Danielsen *et al.* who demonstrated that the erythema and melanin levels of STSG donor site wounds treated with a petrolatum fabric dressing were higher than normal skin 1–3 months post-operation [191]. After 12 months, the erythema levels was still higher than normal skin but the melanin levels decreased to the same as normal skin because melanocytes increase in the first period after injury and decrease in mature scars [191]. However, the melanin and erythema levels of the

biocellulose wound dressing containing silk sericin and PHMB-treated group were significantly less than the control group at healing time and 1 month after wound healing (Figures 7 and 8). Therefore, wounds treated with the biocellulose wound dressing containing silk sericin and PHMB had less inflammation and better scars than the control group. The silk sericin in the biocellulose wound dressing containing silk sericin and PHMB might activate fibroblasts to produce collagen in appropriate amounts, playing an important role in the wound scar process. It also decreased the inflammatory reaction by suppressing COX-2 and nitric oxide genes in the inflammation process [154], leading to a suitable environment for epithelialization and the collagen formation process. In an *in vivo* study, the levels of both inflammatory mediators (IL-1β and TNF-α) in a silk sericin cream-treated group were significantly lower than the normal saline-treated and cream base-treated groups after 7 days [12]. In a clinical study, the researchers reported that sericin cream could reduce pruritus in hemodialysis patients. They found that the sericin cream-treated area had lower melanin and erythema levels than cream base-treated areas after 6 weeks [192]. Accordingly, sericin could reduce skin irritation and skin pigmentation resulting from a post-inflammatory reaction.

TEWL was a measure of the quantity of water that passes from inside a body through the epidermis to the outside environment. It was a reliable, noninvasive method to evaluate the functional barrier recovery of skin. After wound healing, the expected scar should have a low TEWL levels or one close to normal skin. It was measured using a Cutometer® in tewameter mode (Courage+Khazaka electronic GmbH, Germany). If the TEWL values were higher than normal skin, it was characterized as poor quality skin. The TEWL levels of both the biocellulose wound dressing containing silk sericin and PHMB-treated group and the Bactigras® group were significantly higher than normal skin at all-time points. From previous research, the TEWL of STSG donor sites became normal after around 6–13 months [191, 193]. However, the TEWL levels of the biocellulose wound dressing containing silk sericin and PHMB-treated group was significantly less than the Bactigras®-treated group after 1, 3, and 6 months, and continuously decreased 1 to 6 months post-operation (Figure 9). From these results, wounds treated with the biocellulose wound dressing containing silk sericin and PHMB had superior scars to the control group. Silk sericin

was also able to keep wounds moist. It prevented water loss from the stratum corneum and allows water to accumulate in the skin layer. The results in this study agreed with the research of Hasatsri *et al*.. They found that the TEWL index of STSG donor site wounds treated with a bilayered wound dressing containing 1% silk sericin was lower than that of the Bactigras® (control) group after post donor site healing days [34]. Padamwar *et al*. also demonstrated that the TEWL of skin treated with sericin gel was lower than that of non-treated skin [156]. Furthermore, the property of the biocellulose in this study to keep wounds moist was the other important factor for good scar quality. Atiyeh *et al*. found that partial-thickness wounds treated with a moist wound dressing showed lower TEWL and higher scar quality than a semipermeable membrane-occlusive dressing [194].

VSS was used to assess the scar quality with four parameters, vascularity (0–3), pigmentation (0–2), pliability (0–5), and height (0–3) (Appendix G). The maximum total score was 13. A higher VSS score represented poor scar quality. If the total score of the sample was lower than the control, it meant that the scar quality of the sample was better than the control. Overall VSS scores of both dressings were not significantly different. However, the VSS score of the biocellulose wound dressing containing silk sericin and PHMB-treated group was lower than the control group at healing time and 1 month after wound healing because the pigmentation and height scores of the biocellulose wound dressing containing silk sericin and PHMB-treated group were lower than the Bactigras® group (Figures 11, 13, and 14). Moreover, the VSS score of both groups continuously decreased at 3 and 6 months. These results agreed with Barnea *et al.* who studied the efficacy of a moist wound dressing (carboxy-methylcellulose hydrocolloid dressing) and a non-moist wound dressing (paraffin gauze dressing) in STSG donor site treatment. They found that the VSS of the moist wound dressing-treated group was lower than the non-moist wound dressing-treated group after 1, 2, 3, and 12 months [195]. Innes *et al.* also found that the VSS of a moist wound dressing (hydrophilic polyurethane dressing)-treated wound was significantly lower than a non-moist wound dressing (non-adherent nanocrystalline silver-coated dressing)-treated wound 1 and 2 months' post-operation but there was no significant difference after 3 months. The biggest difference in the score was for the pigmentation and vascularity parts [196]. Therefore, from the results

of all parameters, it was concluded that wounds treated with the biocellulose wound dressing containing silk sericin and PHMB had stronger epithelialization than wounds treated with Bactigras®.

The biocellulose wound dressing containing silk sericin and PHMB contained an antimicrobial agent. PHMB was a broad-spectrum antimicrobial agent with high efficacy and low toxicity. It could act as a biocide against aerobic and non-aerobic bacteria through binding between the negatively charged phosphate head groups of the bacterial cell wall and the cationic group of PHMB, and promotes interaction of hexamethylene spacer groups with the hydrophobic interior of the membrane bilayer, leading to membrane fluidity and permeability interference, and cell death [19]. However, this interaction is rarely found in human and animal cells. In this study, there were no signs and symptoms of infection observed in wounds covered with the biocellulose wound dressing containing silk sericin and PHMB or Bactigras®. Biochemistry tests including white blood cell count and neutrophils presented in the normal range at days 0 and 5 after operation (Table 18) because both dressings contained an antimicrobial agent. These results agreed with Daeschlein *et al.* who reported that a PHMB dressing could protect against infection. They reported that no cases of wound infection occurred in secondary burn wounds treated with a 0.04% PHMB solution compared with wounds treated with an undiluted povidone-iodine solution or 1% silver nitrate solution [197]. Eberlein *et al.* [21] found that a PHMB-containing biocellulose dressing had more rapid healing and reduced local wound infection compared to a silver dressing. Andriessen *et al.* also reported that the amount of wound infection of a PHMB solution-treated group was less than Ringer's solution- or saline solution-treated groups in venous leg ulcer treatment [198].

Pain score in this study was assessed by VAS (0 (no pain) to 10 (unbearable pain)). Biocellulose had a cooling property. Moreover, the ultrafine network structures of biocellulose were dense so they are barriers for cell migration into material, which led to pain reduction [6]. Furthermore, silk sericin could reduce an inflammatory reaction by suppressing COX-2 and nitric oxide genes in the inflammation process [154]. Because of these properties of biocellulose and silk sericin, the pain score of the group treated with the biocellulose wound dressing containing silk sericin and PHMB in this study was significantly lower than the

standard treatment at all times (Figure 15). This result agreed with Alvarez *et al.* [7] who studied chronic venous insufficiency and lower leg ulceration patients. Their results also indicated that a biocellulose wound dressing had a significantly lower pain score than a non-adherent petrolatum emulsion-impregnated cellulose acetate gauze treatment. Eberlian *et al.* also reported that the pain score of wounds treated with biocellulose was significantly lower than that of a silver dressing in critically colonized or locally infected wound treatment [21]. Moreover, the biocellulose wound dressing containing PHMB was also demonstrated to significantly reduce pain better and faster than silver sulfadiazine cream in partial-thickness burn patients [33]. Hasatsri *et al.* compared a bilayered wound dressing containing 1% silk sericin and Bactigras® in STSG donor site treatment. They found that the pain score of STSG donor site wounds treated with the bilayered wound dressing containing 1% silk sericin was significantly lower than that of the Bactigras® group 1 to 5 days post-operation [34]. Siritientong *et al.* also found that the pain score of STSG donor site wounds treated with ethyl alcohol-precipitated silk sericin/PVA scaffolds was significantly less than that of Bactigras® 1 to 5 days post-operation [13]. Greater pain reduction increased patient compliance and improved the quality of life of patients.

The biocellulose wound dressing containing silk sericin and PHMB was composed of natural products, biocellulose and silk sericin, that were biocompatible and safe for humans. In this study, no adverse events were observed (Table 19). There were no adverse skin reactions including rash, itching, edema, or papules. Systemic reactions including renal function and hepatic function were normal. These results agreed with Eberlein *et al.*. They studied a biocellulose dressing in critically colonized or locally infected wounds. The results showed that no adverse reactions were observed in either the biocellulose dressing-treated group or the silver dressing (control)-treated group [21]. Alvarez *et al.* also reported that a biocellulose dressing did not significantly induce adverse reactions when compared with a non-adherent petrolatum emulsion-impregnated cellulose acetate gauze in chronic venous insufficiency and lower leg ulceration treatment [7]. No allergic reaction or irritation was found in biocellulose dressing-treated partial-thickness facial burn wounds [8]. Silk sericin was also demonstrated to have no toxicity in full-thickness wound treatment compared with povidone-iodine solution and a cream base without silk

sericin [11]. There were also no adverse effects in silk sericin cream-treated second-degree burn wounds [186]. In STSG donor site treatment, no adverse events were found in a silk sericin-treated group compared with a Bactigras®-treated group [13, 34]. Moreover, PHMB in the dressing is a broad-spectrum antimicrobial agent with high efficacy and low toxicity. It is very friendly to human cells. No adverse reactions or irritation were observed in biocellulose dressing containing PHMB-treated infected wounds [20, 21]. PHMB solution used to treat second-degree burn wounds also had no side effects compared with undiluted povidone-iodine solution or 1% silver nitrate solution [197]. Therefore, this dressing was safe for patients.

The cost of the biocellulose wound dressing containing silk sericin and PHMB was lower than the standard treatment. It could save the overall treatment expense of patients. Patients were easy to approach to use this type of dressing. Moreover, the biocellulose wound dressing containing silk sericin and PHMB was produced from the natural resources of Thailand. It could increase the value of this resource and improve the economy.

Limitations

Phase II clinical study was single blinded study. Population in Phase II clinical study was small sample. Moreover, pain score might be difficult to evaluate because the wounds of both dressings were close. However, the most of patients could assess pain score. For the wound healing time, some patients had misunderstood about dressing detachment. The healing time in this study was the day that the dressing detached by itself with no exudate or air-contact pain. Some patients covered the dressing again when it detached by itself. Therefore, the wound healing time of the biocellulose wound dressing containing silk sericin and PHMB comparing to the control might be the same.

Conclusions

The biocellulose wound dressing containing silk sericin and PHMB was produced from natural materials and contained a wound healing acceleration agent and an antimicrobial agent. For phase I clinical study in healthy volunteers, the biocellulose wound dressing containing silk sericin and PHMB was safe. No significant signs of inflammation or skin irritation were found. In phase II clinical

study, the results demonstrated benefits to STSG donor site wounds. They showed better scar quality and reduced pain compared to the standard treatment (Bactigras®), while there was equal wound healing time because a lot of wound exudate had a direct effect on wounds treated with a low to moderate wound exudate absorption dressing, overcapacity of the wound dressing led to maceration and loss of the dressing's wound healing properties. No signs of infection or adverse events were observed in this study. Moreover, the biocellulose wound dressing containing silk sericin and PHMB was also less expensive than the standard treatment (Bactigras®). Accordingly, the biocellulose wound dressing containing silk sericin and PHMB is suitable for use as an alternative treatment of STSG donor site wounds.

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APPENDICES

Appendix A

แบบบันทึกข้อมูลพื้นฐานของอาสาสมัครเรื่อง ความปลอดภัยของแผ่นปิดแผลไบโอเซลลูโลสผสมโปรตีนกาวไหมเซริซินและสารฆ่าเชื้อ PHMB ในอาสาสมัครสุขภาพดี

ตัวแปร	**หมายเหตุ**
ส่วนที่ 1 ข้อมูลอาสาสมัคร	
ข้อมูลพื้นฐาน	
หมายเลขที่ □□□	
เพศ □1. ชาย □2. หญิง	SEX □
อายุ.....ปี.....เดือน เกิด วันที่.....เดือน.....ปี	AGE □
อาชีพที่ทำเป็นประจำและใช้เวลาเป็นส่วนใหญ่** □1. ว่างงาน □2. แม่บ้าน/พ่อบ้าน □3. เกษตรกร/ประมง □4. ผู้ใช้แรงงาน/รับจ้าง □5. นักเรียน/นักศึกษา □6. ข้าราชการ □7. ตำรวจ/ทหาร □8. พนักงานรัฐวิสาหกิจ □9. พนักงานบริษัท □10. ค้าขาย/ธุรกิจส่วนตัว □11. อื่นๆ (ระบุ).....	OCC □□
น้ำหนักตัว ..... กิโลกรัม***	MEM □□
ความสูง..... เซนติเมตร***	RO □
ประวัติโรคประจำตัว □1. มี ระบุ..... ยาที่ใช้รักษา ..... □2. ไม่มี (ปฏิเสธโรคประจำตัว) *ยาหรือแผ่นแปะที่ใช้ภายใน 2 สัปดาห์ก่อนเข้าร่วมการวิจัย* □ *1. มี ระบุ*..... *ยาที่ใช้รักษา* ..... □ *2. ไม่มี*	
ประวัติการแพ้ (ยา/ อาหารเสริม/ สารเคมี) □ *มี ระบุ*..... □ *ไม่มี*	
ดื่มแอลกอฮอล์**** □ ดื่ม ระบุความถี่ □□ ครั้ง/สัปดาห์ □ ไม่ดื่ม □ เลิกดื่มมาแล้วนาน	Alc□
ดื่มชา/กาแฟ**** □ ดื่ม ระบุความถี่ □□ แก้ว/วัน □ ไม่ดื่ม	Caffeine□
สูบบุหรี่**** □ สูบ/เคยสูบ □□ มวน/วัน นาน.....ปี □ ไม่สูบ □ เลิกดื่มมาแล้วนาน	Smoking□

Appendix B

ประเมินอาการไม่พึงประสงค์โดยแพทย์ผิวหนัง

Erythema scale:

This scale is used only for grading degree of erythema (redness). A score on this scale will be assigned following every application of a patch.

0 No visible erythema.

1 Mild erythema (faint pink to definite pink).

2 Moderate erythema (definite redness).

3 Severe erythema (very intense redness).

Designations for Elevated Responses:

Edema, papules, vesicles, and bullae, if present, are graded as independent responses.

E Edema - definite swelling. (0-4 ; no edema – severe edema)

P Papules - many small, red, solid elevations; surface of reaction has granular feeling.

V Vesicles - small, circumscribed elevations having translucent surfaces so that fluid is visible (blisterlike). Vesicles are no larger than 0.5 cm in diameter.

B Bullae - vesicles with a diameter > 0.5 cm; vesicles may coalesce to form one or a few large blisters that fill the patch site.

เกณฑ์คะแนนประเมินอาการไม่พึงประสงค์โดยแพทย์ผิวหนัง เลขที่อาสาสมัคร □□□

ตำแหน่งที่ 1

ลักษณะ	คะแนนประเมินสภาพผิวหนัง			คะแนนประเมิน
	ครั้งที่ 1	ครั้งที่ 2	ครั้งที่ 3	
Erythema				0-3
Edema				0-4
Papules				มี/ไม่มี
Vesicles				มี/ไม่มี
Bullae				มี/ไม่มี

ตำแหน่งที่ 2

ลักษณะ	คะแนนประเมินสภาพผิวหนัง			คะแนนประเมิน
	ครั้งที่ 1	ครั้งที่ 2	ครั้งที่ 3	
Erythema				0-3
Edema				0-4
Papules				มี/ไม่มี
Vesicles				มี/ไม่มี
Bullae				มี/ไม่มี
Bullae				มี/ไม่มี

Appendix C

แบบฟอร์มการวัดความแดงของผิวหนังด้วยเครื่อง Cutometer® ด้วยโหมด Mexameter

วันที่ ...../...../..... เลขที่อาสาสมัคร □□□

ตำแหน่งที่ ☐ **1** ☐ **2** ☐ **3** ☐ **4**

ความแดงของ ผิวหนัง (วัดตำแหน่งละ 10 ซ้ำ)	Baseline	ครั้งที่ 1	ครั้งที่ 2	ครั้งที่ 3
1				
2				
3				
4				
5				
6				
7				
8				
9				
10				
Mean				
SD				

Appendix D

Demographic data

Title: Clinical efficacy of biocellulose wound dressing containing silk sericin and PHMB for STSG donor sites

Patient information and demographics		
Patient code:_____	Date:_____/_____/_____	Sex: ☐ Male ☐ Female
Date of birth:_____/_____/_____	Weight (kg):_____	Height (cm): _____
Comorbids:_____		
Current medications:_____		
Smoking: ☐ Yes _____ cigarettes/day ☐ Used to smoke but quit in_____ ☐ No		
Allergies:_____		
Treatment information		
Diagnosis:_____		
Size of STSG donor site (cm^2):_____		
Area of STSG donor site _____		
Date of admit:____/___/___	Date of surgery:____/___/___	Date of discharge:___/___/__

Medications/ Nutritions	Dose	Regimen	Date started
1. 2. 3. 4. 5. 6. 7. 8. 9. 10.			

Appendix E

Follow up report

Title: Clinical efficacy of biocellulose wound dressing containing silk sericin and PHMB for STSG donor sites

A.(dressing name)_____ **B.** (dressing name)_____

Patient code:_____ Date:_____/_____/_____

Wound Healing Time

A.	**B.**
- Area_____ - Secondary dressing change day _____ - Exudates ☐ Yes day☐__ No - Wound size (cm^2) _____ - Healing ☐Yes day____ ☐ No	- Area_____ - Secondary dressing change day ___ - Exudates ☐Yes day____ ☐No - Wound size (cm^2) _____ - Healing ☐ Yes day____ ☐ No

Signs of infection

A.	**B.**
Pus ☐ Yes_____ ☐ No - Swelling, redness, bad odor Others_____ Swab result _____	Pus ☐ Yes_____ ☐ No - Swelling, redness, bad odor Others_____ Swab result _____

Visual analogue scale

A.

No pain |—|—|—|—|—|—|—|—|—|—| Unbearable

B.

No pain |—|—|—|—|—|—|—|—|—|—| Unbearable

Pain medication name_____

☐ Yes _____dose

☐ No

Pain medication name_____

☐ Yes _____dose

☐ No

Pain medication name_____

☐ Yes _____dose

☐ No

Vital signs	
Heart rate (beats per minute):_____	Respiration (breaths per minute):_____
Blood pressure (mmHg):_____	Temperature (˚C):_____

Blood chemistry		
BUN:_____	Serum creatinine:_____	
AST:_____	ALT:_____	
WBC:_____	RBC:_____	Neutrophils:_____

Patient's medication change

Medications/ Nutritions	Dose	Regimen	Date started
1. 2. 3. 4. 5. 6. 7. 8. 9. 10.			

Appendix F

Adverse event report

Naranjo's algorithm

Question	Yes	No	Don't Know
1. Are there previous conclusive reports on this reaction?	+1	0	0
2. Did the adverse event appear the suspected drug was administered?	+2	-1	0
3. Did the adverse reaction improve when the drug was discontinued or a specific antagonist was administered?	+1	0	0
4. Did the adverse reaction reappear when the drug was readministered?	+2	-1	0
5. Are there alternative causes (other than the drug) that could on their own have caused the reaction?	-1	+2	0
6. Did the reaction reappear when a placebo was given?	-1	+1	0
7. Was the drug detected in the blood (or other fluids) in concentrations known to be toxic?	+1	0	0
8. Was the reaction more severe when the dose was increased, or less severe when the dose was decreased?	+1	0	0
9. Did the patient have a similar reaction to the same or similar drugs in any previous exposure?	+1	0	0
10. Was the adverse event confirmed by any objective evidence?	+1	0	0
Total Score			

The total score calculated from this table defines the category an adverse reaction belongs to. The categories are defined as follows:

Definite (Certain) (total score>9)

Probable (total score 5-8)

Possible (total score1-4)

Doubtful (Unlikely) (total score <1)

Sign……..

(Investigator)

Date…….

Appendix G

Vancouver scar scale report

Title: Clinical efficacy of biocellulose wound dressing containing silk sericin and PHMB and Bactigras® in the treatment of STSG donor site

Patient code: _____

A. (dressing name)_____

Parameters	**Description**	**Points**			
		F/U 1	**F/U 2**	**F/U 3**	**F/U 4**
		____	____	_____	_____
Vascularity	Normal	0	0	0	0
	Pink (slightly increased in local blood supply)	1	1	1	1
	Red (significant increase in local blood supply)	2	2	2	2
	Purple (excessive local blood supply)	3	3	3	3
Pigmentation	Normal	0	0	0	0
	Hypopigmentation	1	1	1	1
	Hyperpigmentation	2	2	2	2
Pliability (Elasticity)	Normal	0	0	0	0
	Supple (flexible with minimal resistance)	1	1	1	1
	Yielding (giving way to pressure)	2	2	2	2
	Firm (solid/inflexible, not easy to move, resistant to manual pressure)	3	3	3	3
	Banding (rope-like/blanches with extension of scar, does not limit range of motion)	4	4	4	4
	Contracture (permanent shortening of scar, producing deformity or distortion; limits range of motion)	5	5	5	5
Height	Normal (flat)	0	0	0	0
	> 0 and < 2mm	1	1	1	1
	> 2 and < 5mm	2	2	2	2
	> 5mm	3	3	3	3
Total score		/13	/13	/13	/13

B. (dressing name)_____

Parameters	Description	Points			
		F/U 1	F/U 2	F/U 3	F/U 4
		____	____	_____	_____
Vascularity	Normal	0	0	0	0
	Pink (slightly increased in local blood supply)	1	1	1	1
	Red (significant increase in local blood supply)	2	2	2	2
	Purple (excessive local blood supply)	3	3	3	3
Pigmentation	Normal	0	0	0	0
	Hypopigmentation	1	1	1	1
	Hyperpigmentation	2	2	2	2
Pliability (Elasticity)	Normal	0	0	0	0
	Supple (flexible with minimal resistance)	1	1	1	1
	Yielding (giving way to pressure)	2	2	2	2
	Firm (solid/inflexible, not easy to move, resistant to manual pressure)	3	3	3	3
	Banding (rope-like/blanches with extension of scar, does not limit range of motion)	4	4	4	4
	Contracture (permanent shortening of scar, producing deformity or distortion; limits range of motion)	5	5	5	5
Height	Normal (flat)	0	0	0	0
	> 0 and < 2mm	1	1	1	1
	> 2 and < 5mm	2	2	2	2
	> 5mm	3	3	3	3
Total score		/13	/13	/13	/13

Appendix H

Preliminary materials, methods and results

1. Preliminary materials and methods

1.1 Releasing test

The biocellulose wound dressing containing silk sericin (1%) and PHMB (0.3%) was cut to a size of 1 × 1 × 0.1 cm^3 and placed in triplicate in a vessel containing 3 ml of phosphate-buffered saline solution (PBS, pH 7.4) at 37 °C. The PBS solutions were collected at pre-determined time points (0.5, 2, 4, 8, 24, 48, 72 and 168 hours) and shaken before measurement. In the silk sericin case, the BCA protein assay kit (Pierce, Rockford, IL, USA) was used to measure the released amount of silk sericin protein. The absorbance of the solution was measured with an UV/VIS spectrometer at a wavelength of 562 nm. The amount of silk sericin was determined from a standard curve prepared from different concentrations of bovine serum albumin. For PHMB, the solution absorbance was measured with the same spectrometer at a wavelength of 235.5 nm. The concentrations of released PHMB at different times were calculated by evaluating the solution absorbance against a standard curve, prepared from different known PHMB concentrations.

1.2 *In vivo* safety test

The animal experiments were performed according to Chulalongkorn University Animal Care and Use Committee (CUACUC 13/57) under standard sterile conditions. Briefly, female Wistar rats (8 weeks old, weight (200–300 g) were anesthetized, shaved the hair, and disinfected with 70 % ethyl alcohol. A 1 cm skin incision was made to form pockets in the subcutaneous tissue. Then, the dressing sample (biocellulose wound dressing containing silk sericin (1%) and PHMB (0.3%) and Bactigras®) was inserted into each pocket. The wound was sutured with 6-0 prolene and disinfected with povidone iodine topical antiseptic solution. After 7, 14, 21, and 28 days of implantation, the rats were killed. The samples and surrounding tissue were retrieved, fixed with 10 % formalin solution, and embedded in paraffin. The paraffin embedded

samples were sectioned (4 μm) and immunohistochemical staining was proceeded as follows. The sections were deparaffinized in xylene and rehydrated prior to immunohistochemical staining. Endogenous peroxidase of the sample sections was quenched with 1 %v/v hydrogen peroxide in methanol, washed with 0.2 %v/v Tween in phosphate-buffered saline (0.2 % T-PBS), and blocked with 100 % FBS for 40 min. The sections were then incubated with mouse monoclonal anti FOXP3+ antibody (Santa Cruz, USA) diluted in PBS + 1 %v/v FBS (Vector, USA, S1000) for 60 min, washed with 0.2 % T-PBS or three times, and incubated with labeled polymer HRP antimouse/rabbit EnVision kit (Dako, Denmark, K5007) for 60 min according to the manufacturer's instruction. For visualization, the sections were stained with diaminobenzidine staining (DAB, DAKO, Denmark, K3468) for 3 min, washed, counterstained with hematoxylin, and permanently mounted with DPX. Inflammatory responses to the dressings implanted including foreign body giant cells, fatty infiltration, fibrosis, and degranulation of mast cells were evaluated.

1.3 *In vivo* efficacy test

The experimental protocol of efficacy test was approved by Mahidol University Animal Care and Use Committee (MU-ACUC) in 2014. Twenty-four Wistar rats (8 weeks old, weight 300 ± 20 g) were anesthetized, shaved the hair, and disinfected with 70 % ethyl alcohol. Then, the full thickness wound (1.5 x 1.5 cm^2) was created on skin dorsal to subcutaneous depth. One rat received two wounds on left and right sides. The wounds were randomly covered with biocellulose wound dressing containing silk sericin (1%) and PHMB (0.3%) and Bactigras®. All wounds were covered with transparent dressing (Tegaderm™) as a secondary dressing and adherent wrap (Coban™) as a tertiary dressing. The rats received 0.5–1.0 mg/kg of Tramadol via subcutaneous injection every day for 3 days. At days 3, 7, 14 and 21, wound size was evaluated by Visitrak™ digital device and wound infection was assessed by swab test. Area fraction of collagen was semi-quantitative measured from those acquired images using ImageJ program, NIH. Briefly,

color images were transformed to gray scale and located collagen bundle as interested area. Thus, the area fraction of positive collagen was determined as the percentage/image.

2. Preliminary results

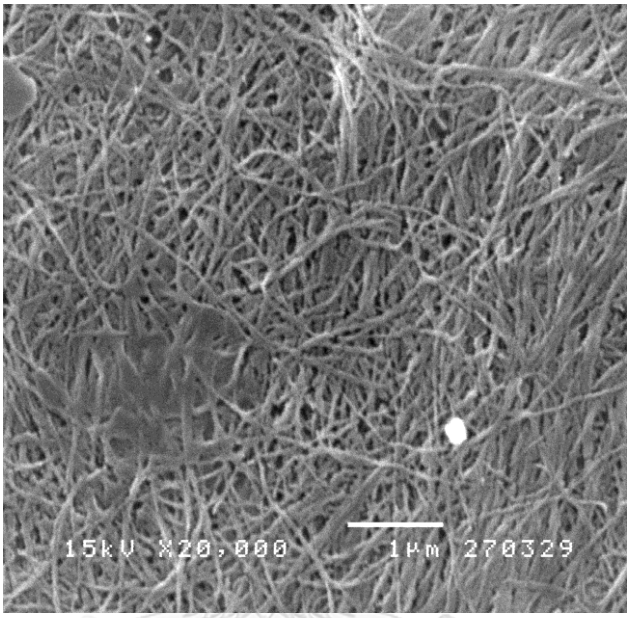

Figure 1H The structure of the biocellulose containing silk sericin and PHMB and biocellulose wound dressing using scanning electron microscope (SEM)

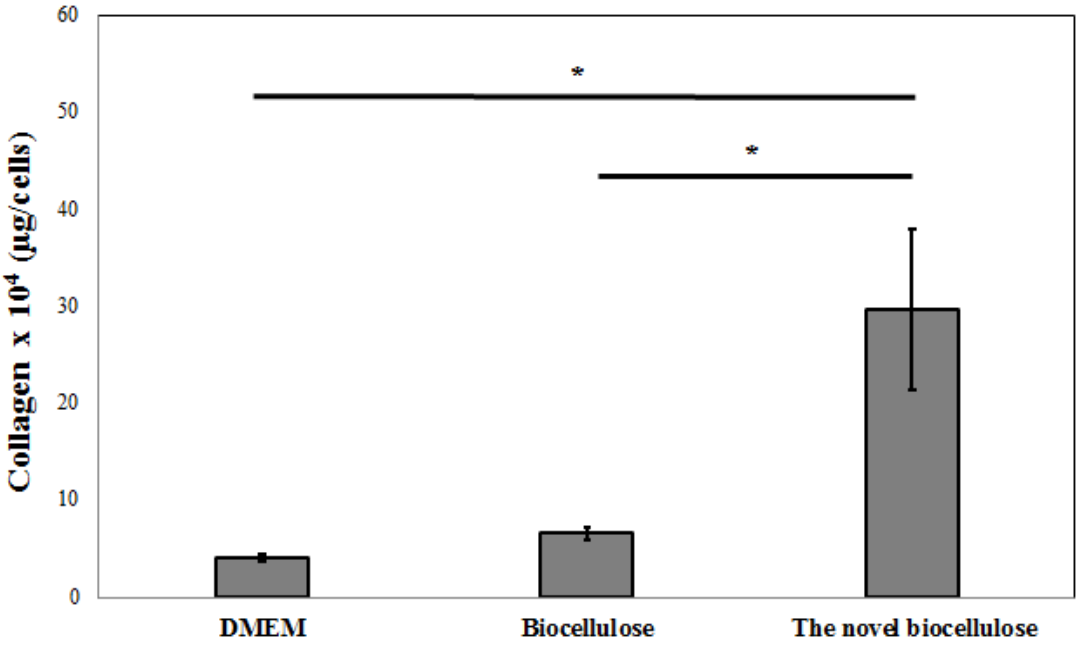

Figure 2H The amount of collagen type 1 in released solution of the biocellulose containing silk sericin and PHMB and biocellulose wound dressing (* indicates a statistical difference ($p < 0.05$))

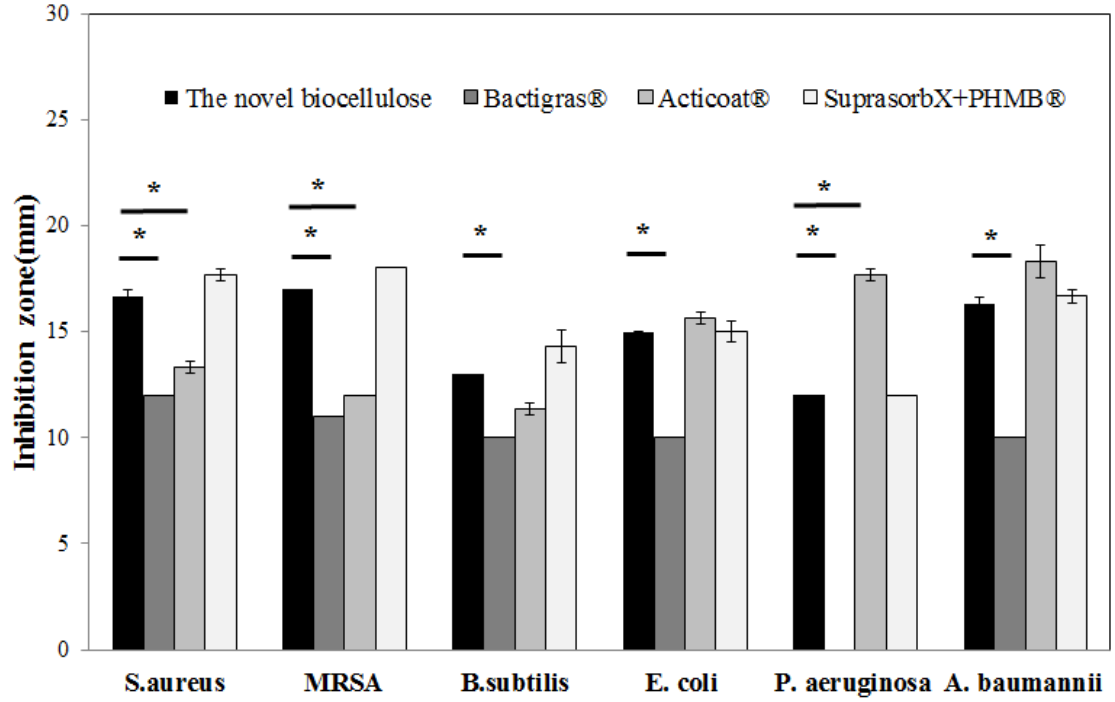

Figure 3H A comparison of the antimicrobial activity of the biocellulose containing silk sericin and PHMB with commerically available antimicrobial wound dressings (Bactigras®, Acticoat® and SuprasorbX+PHMB®) against all tested bacteria (*S.*

aureus, MRSA, *B. subtilis*, *E. coli*, *P. aeruginosa*, and *A. baumannii*), 24 h after placing the biocellulose containing silk sericin and PHMB on bacteria-loaded MH agar plates which were cultured at 37ºC (* indicates a statistically significant difference ($p < 0.05$) between the biocellulose containing silk sericin and PHMB and other commercial dressings).

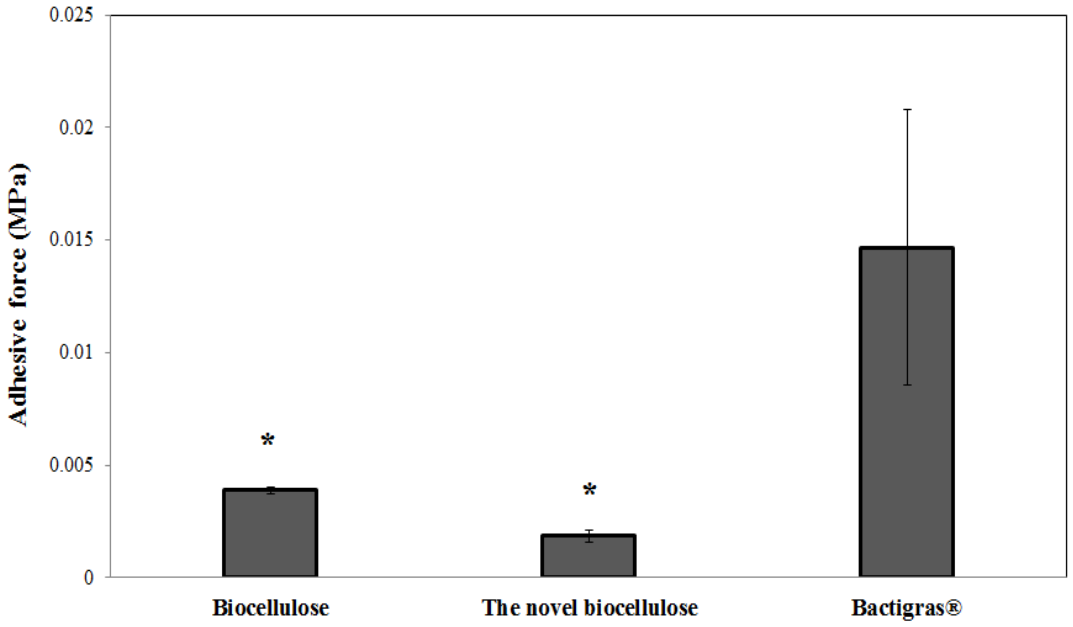

Figure 4H Adhesive force applied to peel off the biocellulose containing silk sericin and PHMB, biocellulose dressings, and Bactigras® from wound on porcine skin. (* indicates a statistical difference ($p < 0.05$))

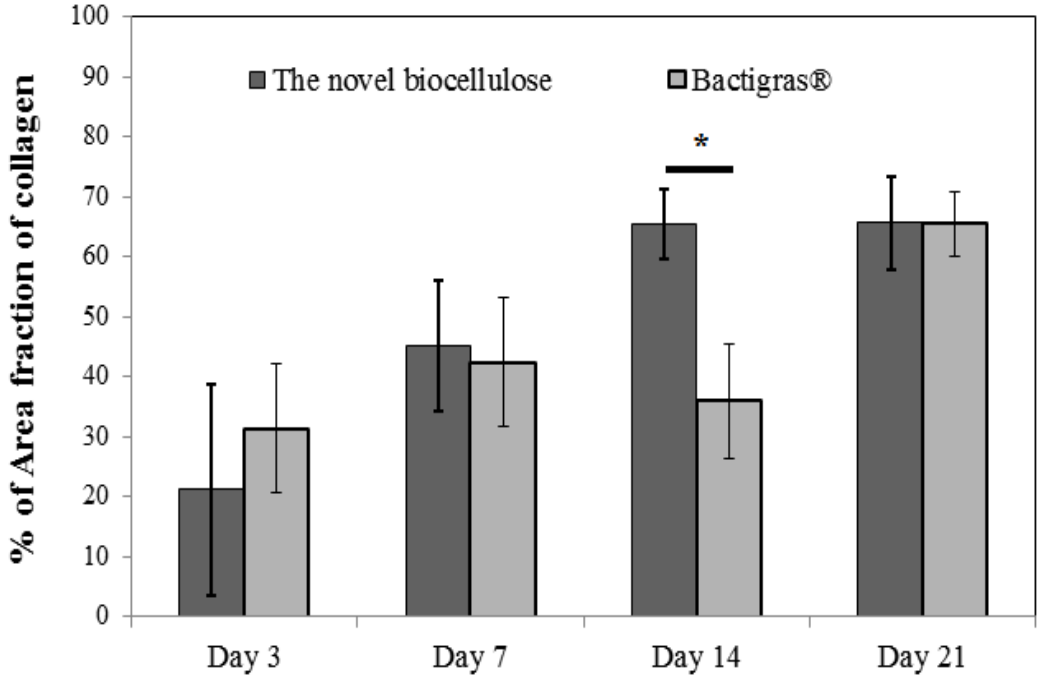

Figure 5H The percentage of area fraction of collagen of biocellulose containing silk sericin and PHMB and Bactigras® (control) on days 3, 7, 14, and 21 (* significant difference when compared with the Bactigras® ($p < 0.05$))

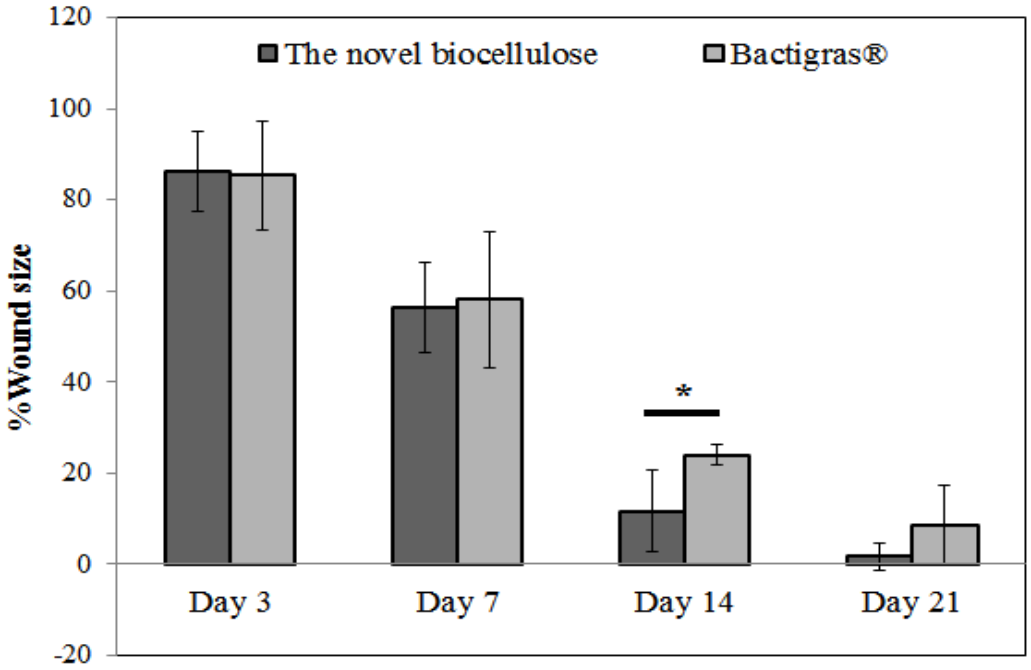

Figure 6H The percentage of wounds size of biocellulose containing silk sericin and PHMB and Bactigras® (control) on days 3, 7, 14, and 21 (* significant difference when compared with the Bactigras® ($p < 0.05$)).

Appendix I

Detail of result documents

Table 1I Healing time of STSG donor site wound covered with biocellulose wound dressing containing silk sericin and PHMB (BC/SS/PHMB) and Bactigras®

No	Healing time (days)	
	BC/SS/PHMB	**Bactigras®**
1	16	16
2	22	22
3	22	22
4	18	18
5	20	20
6	20	19
7	19	18
8	19	19
9	25	25
10	31	31
11	25	25
12	21	21
13	13	13
14	11	11
15	21	21
16	15	15
17	15	15
18	17	17
19	17	17
20	17	17
21	17	17
22	23	23
23	21	21
24	13	13

No	Healing time (days)	
	BC/SS/PHMB	**Bactigras®**
25	13	13
26	13	13
27	13	13
28	20	20
29	27	27
30	27	27
31	27	27
32	27	27

Table 2I Melanin levels of STSG donor site wound covered with biocellulose wound dressing containing silk sericin and PHMB (BC/SS/PHMB) and Bactigras® at healing day, 1, 3, and 6 months

No	Healing day			1 month			3 months			6 months		
	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®
1	181.10	86.60	91.50	165.95	73.50	129.65	168.25	301.50	303.75	265.30	411.05	426.20
2	268.50	33.75	118.80	239.95	57.75	86.90	236.30	193.30	135.00	333.30	267.20	227.80
3	247.05	40.50	91.20	208.30	43.20	60.90	215.80	107.30	156.90	283.70	179.20	183.40
4	293.55	110.15	128.50	286.80	110.80	192.80	293.10	419.53	413.05	443.80	557.50	608.80
5	184.85	21.75	100.85	180.45	147.90	190.40	367.15	420.05	407.40	247.25	216.95	248.35
6	112.40	35.65	23.95	133.95	79.35	148.35	328.55	399.05	395.50	188.10	232.80	252.10
7	225.40	37.30	126.40	235.70	145.95	249.95	418.05	438.15	480.15	238.85	260.25	277.75
8	224.30	71.50	51.75	311.30	272.80	207.00	434.55	412.65	372.40	275.30	125.35	91.50
9	182.35	159.35	142.30	180.10	225.60	233.45	334.65	503.35	520.55	242.85	336.60	387.35
10	344.55	184.40	265.89	482.55	413.40	384.20	444.40	352.25	369.00	327.95	281.80	291.20
11	299.20	257.25	267.65	323.90	221.85	215.40	439.50	492.45	469.60	339.75	246.75	196.60
12	419.10	275.30	258.10	442.30	322.50	234.85	400.65	371.00	378.15	361.23	454.55	446.70
13	477.05	248.15	307.45	462.85	308.80	411.10	366.30	538.55	458.65	301.60	393.20	439.60
14	340.20	148.25	269.35	303.05	278.65	383.65	246.70	358.05	401.95	220.05	298.80	329.00
15	425.40	245.10	281.25	434.00	303.55	315.65	406.20	332.95	360.70	351.45	423.75	447.00

No	Healing day			1 month			3 months			6 months		
	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®
16	373.40	279.00	315.80	344.05	343.65	386.90	260.65	308.60	286.00	209.25	303.05	234.00
17	387.60	214.25	272.60	350.20	347.70	338.20	250.75	360.70	283.95	232.70	332.30	261.50
18	360.25	325.25	311.50	415.20	421.05	451.60	312.60	488.35	500.95	292.85	407.70	394.70
19	411.50	199.95	324.65	418.45	364.75	423.00	306.40	538.10	546.60	312.20	496.05	473.45
20	336.65	176.00	323.65	302.95	319.25	409.15	308.45	433.95	503.90	313.35	397.15	369.05
21	496.95	267.90	291.15	529.40	490.10	497.55	405.00	523.25	437.00	380.80	544.55	415.00
22	360.65	255.15	312.20	322.65	314.50	347.55	232.70	428.60	447.60	242.70	349.50	359.95
23	262.30	194.60	245.30	233.80	214.05	251.65	122.15	193.63	187.13	108.65	158.40	166.10
24	449.55	155.75	155.20	438.45	376.60	422.10	337.35	242.80	219.05	297.25	255.30	199.90
25	433.35	161.30	214.75	440.70	357.00	455.50	329.70	385.05	328.60	305.55	248.80	243.15
26	472.20	124.25	187.75	455.15	351.55	357.45	316.70	173.85	300.60	331.05	164.20	168.80
27	455.30	124.55	225.05	463.35	356.80	415.15	373.10	363.70	467.30	352.55	256.05	342.30
28	514.20	251.50	288.45	383.60	408.40	440.50	399.80	545.00	512.00	393.20	520.55	492.15
29	419.55	137.20	297.85	426.40	214.45	274.50	410.95	217.75	221.30	391.25	358.40	312.70
30	414.50	146.40	186.30	425.20	192.35	195.60	410.55	156.40	144.35	394.25	357.40	320.80
31	364.20	131.90	185.10	389.20	178.50	190.35	372.50	149.20	159.10	360.85	341.30	399.45
32	369.65	210.75	274.90	409.40	222.70	232.80	371.20	230.60	197.50	371.90	408.75	425.40

Table 3I Erythema levels of STSG donor site wound covered with biocellulose wound dressing containing silk sericin and PHMB (BC/SS/PHMB) and Bactigras® at healing day, 1, 3, and 6 months

No	Healing day			1 month			3 months			6 months		
	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®
1	202.40	543.60	615.40	193.55	652.80	675.65	211.00	445.30	492.95	213.80	487.30	508.55
2	345.20	568.35	589.25	286.15	522.70	511.35	311.30	463.60	362.80	285.80	287.90	227.80
3	303.60	558.45	518.95	261.05	463.60	525.45	285.80	348.80	368.05	254.45	192.95	171.15
4	350.00	700.65	582.05	312.95	510.60	451.65	354.35	460.48	418.00	380.30	422.65	464.25
5	190.25	492.50	584.65	193.10	319.45	435.60	174.80	325.10	298.35	180.15	218.25	248.35
6	186.80	681.95	722.10	252.75	771.25	799.15	255.80	575.05	569.80	246.05	386.10	381.25
7	244.00	482.45	450.40	320.30	541.75	512.70	242.00	376.75	367.30	232.30	288.35	265.55
8	257.25	474.10	433.20	386.00	563.55	525.05	359.85	522.40	466.05	281.80	501.35	582.35
9	182.60	409.80	492.05	188.05	430.60	443.50	161.20	381.70	395.65	198.80	341.25	357.20
10	381.65	664.90	771.01	388.60	625.55	684.05	337.05	527.50	544.70	350.00	499.05	474.65
11	400.25	275.65	674.15	423.95	467.25	497.85	356.95	492.45	469.60	337.70	472.95	493.70
12	259.25	662.15	737.85	291.65	463.80	577.45	335.30	436.15	508.85	302.23	422.60	442.80
13	322.45	475.20	575.85	308.80	430.45	540.80	259.75	459.35	447.75	262.05	398.65	438.35
14	200.50	725.30	581.75	188.80	526.50	669.05	230.05	525.60	504.90	195.50	459.55	401.25

No	Healing day			1 month			3 months			6 months		
	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®
15	256.85	619.40	795.10	301.30	502.40	503.50	359.10	416.85	457.20	294.55	410.25	438.80
16	221.95	378.85	519.95	209.90	502.70	546.05	225.15	418.50	393.80	180.20	355.25	266.15
17	219.30	503.45	545.55	214.90	453.35	617.20	228.65	449.45	421.60	213.30	388.80	360.65
18	188.95	325.25	328.95	234.90	292.00	327.30	300.65	470.35	512.60	378.05	470.25	498.60
19	267.50	482.05	440.80	248.70	314.20	335.80	305.75	522.75	519.30	338.85	517.55	519.90
20	205.05	405.20	462.65	191.15	356.85	489.90	314.10	474.30	498.75	348.65	462.00	438.65
21	377.55	639.55	629.45	393.80	581.65	609.50	385.10	568.20	548.60	363.80	485.85	471.10
22	255.50	465.20	493.40	227.85	442.05	433.65	268.80	447.30	405.10	268.00	339.90	340.30
23	93.30	496.00	678.00	95.15	503.25	612.15	114.05	479.04	512.46	77.60	275.70	298.55
24	313.70	528.70	542.95	276.50	402.15	480.05	308.75	352.40	345.95	264.30	315.00	324.75
25	306.40	562.25	535.75	290.85	511.70	621.05	306.00	465.55	440.75	293.30	3556.40	330.00
26	331.15	532.95	515.80	291.00	377.75	438.05	285.35	319.75	418.15	299.30	267.65	265.05
27	311.55	548.50	550.45	294.45	448.95	522.00	322.60	431.35	453.75	300.20	350.60	389.55
28	353.80	514.05	632.65	319.55	570.60	588.10	336.60	501.05	485.30	301.70	479.20	453.50
29	316.40	488.85	724.35	365.60	427.30	497.20	342.35	472.90	531.75	397.70	514.95	486.20
30	298.40	399.10	630.70	345.20	280.35	463.40	355.40	442.00	538.85	405.15	460.20	459.45

No	Healing day			1 month			3 months			6 months		
	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®
31	282.90	411.50	566.55	332.80	450.55	469.75	331.50	476.80	574.60	382.20	450.45	496.85
32	285.60	618.30	485.80	363.60	487.85	378.45	325.70	462.50	514.60	391.30	485.95	496.15

Table 4I TEWL levels of STSG donor site wound covered with biocellulose wound dressing containing silk sericin and PHMB (BC/SS/PHMB) and Bactigras® at healing day, 1, 3, and 6 months

No	Healing day			1 month			3 months			6 months		
	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®
1	1.66	11.93	9.79	4.63	20.95	19.22	1.47	6.86	7.29	5.38	7.41	8.10
2	3.88	19.15	12.81	2.97	15.11	19.26	2.14	6.80	5.95	2.00	5.17	5.48
3	4.27	11.64	16.84	6.01	21.58	18.37	4.09	6.68	9.18	5.75	5.21	6.54
4	4.94	22.88	18.32	5.24	30.34	31.28	3.64	8.83	5.92	2.21	4.54	4.64
5	3.33	16.39	21.71	4.32	6.53	8.12	1.59	1.98	3.37	2.89	1.90	3.88
6	3.35	19.26	18.09	1.96	13.36	17.02	1.35	11.80	15.27	6.53	6.75	11.18
7	2.81	22.81	22.25	3.68	17.06	22.72	1.18	5.19	7.15	2.23	5.36	6.11
8	2.02	20.16	15.31	4.83	5.51	15.60	1.12	6.86	8.12	5.68	18.73	23.20
9	2.08	8.17	11.60	0.80	9.78	16.27	2.56	10.00	13.21	3.44	4.99	6.80
10	2.15	7.83	11.92	1.46	7.17	8.75	2.03	5.47	7.93	4.67	7.66	9.51
11	2.31	10.84	17.31	2.36	11.56	14.05	5.06	6.83	8.27	4.03	11.22	12.60
12	4.21	18.70	21.47	0.79	11.40	18.91	11.35	32.04	42.64	10.85	13.12	15.88
13	3.47	16.72	27.04	2.80	23.31	30.95	4.40	17.33	25.37	7.02	10.58	10.41
14	2.72	25.63	24.79	0.96	8.67	12.83	2.75	10.75	11.01	26.89	32.77	35.71
15	3.33	12.13	20.46	2.65	11.35	13.49	13.36	21.64	26.42	11.86	13.51	14.77

No	Healing day			1 month			3 months			6 months		
	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®	Normal skin	BC/SS/ PHMB	Bacti-gras®
16	1.55	16.70	19.34	1.83	10.82	12.79	2.88	8.67	13.50	2.97	7.28	7.26
17	4.20	19.86	16.38	4.78	10.28	11.72	9.22	13.17	12.55	5.27	6.84	6.11
18	1.87	15.43	17.04	7.14	22.26	25.10	4.00	13.42	13.26	7.70	7.11	7.48
19	4.37	17.24	19.95	7.89	21.87	26.95	6.54	18.30	25.67	6.87	10.13	13.46
20	4.77	16.42	20.87	1.57	12.78	19.10	7.46	12.14	14.51	8.04	10.00	10.17
21	1.75	17.45	19.64	1.97	16.95	19.33	2.84	7.28	7.79	4.94	7.71	7.59
22	1.56	11.27	13.34	1.22	8.57	11.07	3.04	12.18	14.43	1.61	2.42	3.08
23	0.98	16.71	20.10	1.13	11.58	14.03	9.25	17.53	18.40	34.31	14.86	22.14
24	3.18	30.43	26.99	2.54	13.20	21.87	4.05	9.04	10.35	2.16	5.74	9.74
25	6.55	35.13	23.91	7.60	14.69	17.47	5.94	9.52	9.95	5.47	8.04	8.71
26	4.22	20.48	23.53	6.27	17.35	20.67	5.89	9.82	12.48	7.31	10.49	11.07
27	6.82	24.28	20.78	4.94	15.02	17.51	6.89	11.54	13.66	4.51	6.09	8.16
28	1.13	12.97	18.27	1.18	15.22	13.22	4.41	4.37	6.71	5.67	5.43	7.73
29	1.51	16.17	23.66	1.38	12.95	16.69	2.01	7.25	10.00	12.25	11.33	12.08
30	2.42	25.49	19.21	3.29	12.48	12.49	3.96	7.50	8.89	17.72	19.30	25.68
31	3.26	18.63	19.16	1.94	12.47	14.21	3.70	10.14	10.34	9.97	15.81	19.93
32	5.01	34.79	36.25	2.73	13.25	12.31	3.26	12.28	16.03	13.04	16.51	17.13

Table 5I VSS of STSG donor site wound covered with biocellulose wound dressing containing silk sericin and PHMB (BC/SS/PHMB) and Bactigras® at healing day, 1, 3, and 6 months

No	Healing day		1 month		3 months		6 months	
	BC/SS/PHMB	Bactigras®	BC/SS/PHMB	Bactigras®	BC/SS/PHMB	Bactigras®	BC/SS/PHMB	Bactigras®
1	1.5	2.0	2.0	7.5	4.0	4.5	6.5	7.5
2	1.0	0.5	0.5	2.5	2.0	0.5	0.5	1.0
3	1.5	1.5	1.5	1.0	1.5	1.0	0.5	1.0
4	1.5	1.5	1.5	1.5	2.0	0.0	1.0	1.0
5	2.0	5.5	5.5	5.0	1.5	1.5	0.5	0.5
6	2.0	2.5	2.5	2.0	3.5	3.0	3.5	3.5
7	1.0	1.5	1.5	3.0	0.0	0.0	1.0	1.0
8	1.0	0.5	0.5	2.5	3.0	1.5	3.0	3.0
9	3.0	2.5	2.5	2.0	2.0	2.0	1.0	2.0
10	3.0	2.5	2.5	1.0	1.0	0.5	1.0	1.0
11	3.5	4.0	4.0	2.5	3.0	1.5	1.5	3.0
12	2.0	1.5	1.5	1.5	5.0	3.0	1.0	2.0
13	2.0	2.0	2.0	1.5	2.0	1.5	1.0	2.0
14	2.0	2.0	2.0	3.5	5.5	5.5	3.0	2.0
15	3.0	3.0	3.0	2.5	5.5	5.5	1.5	2.0

No	Healing day		1 month		3 months		6 months	
	BC/SS/PHMB	Bactigras®	BC/SS/PHMB	Bactigras®	BC/SS/PHMB	Bactigras®	BC/SS/PHMB	Bactigras®
16	2.5	3.0	3.0	2.5	2.0	2.0	1.5	2.0
17	2.0	2.5	2.5	2.5	2.0	2.0	1.5	1.0
18	3.0	3.0	3.0	2.0	2.0	2.5	2.0	0.0
19	2.0	3.0	3.0	3.5	2.0	2.0	2.0	2.0
20	1.5	1.5	1.5	2.0	2.0	2.0	2.0	0.0
21	3.0	2.5	2.5	1.5	2.0	0.0	2.0	0.0
22	3.5	3.0	3.0	3.0	4.0	3.5	3.0	3.0
23	2.0	2.5	2.5	2.0	1.0	1.0	1.0	1.0
24	1.5	1.0	1.0	2.0	1.0	1.0	1.0	1.0
25	1.0	1.0	1.0	4.5	2.0	1.0	1.0	1.0
26	1.5	1.5	1.5	1.0	1.0	1.0	1.0	1.0
27	3.0	2.5	2.5	3.0	3.0	3.0	2.0	2.0
28	3.0	4.5	4.5	2.5	3.0	3.0	4.0	1.0
29	2.0	3.0	3.0	3.5	2.0	2.0	2.0	2.0
30	1.0	1.0	1.0	3.0	3.0	2.0	4.0	1.0
31	2.5	3.0	3.0	3.5	4.0	4.0	4.0	5.0
32	4.0	4.5	4.5	3.0	3.0	3.0	4.0	4.0

Table 6I Pain score of STSG donor site wound covered with biocellulose wound dressing containing silk sericin and PHMB (BC/SS/PHMB) and Bactigras® at day 1-5

No	Day 1		Day 2		Day 3		Day 4		Day 5	
	BC/SS/ PHMB	Bacti-gras®	BC/SS/ PHMB	Bacti-gras®	BC/SS PHMB	Bacti-gras®	BC/SS/ PHMB	Bacti-gras®	BC/SS/ PHMB	Bacti-gras®
1	6	9	6	8	4	7	2	6	2	6
2	8	8	8	7	6	6	5	5	5	5
3	4	7	4	6	2	5	1	4	1	4
4	3	5	3	4	2	4	0	2	1	3
5	4	8	4	7	2	6	0	3	0	1
6	3	6	3	5	1	4	0	3	0	2
7	4	8	4	7	2	6	0	3	0	2
8	3	5	3	4	1	3	0	2	0	2
9	5	5	5	4	2	2	1	1	0	1
10	7	9	8	9	3	5	2	4	2	3
11	5	5	4	3	1	2	0	1	0	0
12	7	7	7	6	5	5	4	4	4	4
13	3	6	3	3	1	6	0	0	0	0

No	Day 1		Day 2		Day 3		Day 4		Day 5	
	BC/SS/ PHMB	Bacti-gras®	BC/SS/ PHMB	Bacti-gras®	BC/SS PHMB	Bacti-gras®	BC/SS/ PHMB	Bacti-gras®	BC/SS/ PHMB	Bacti-gras®
14	6	8	3	5	1	3	0	2	0	4
15	7	7	7	6	5	5	4	4	4	4
16	6	7	4	5	1	4	0	3	0	3
17	6	7	4	5	1	4	0	3	0	3
18	4	4	2	7	1	4	0	2	0	2
19	4	4	2	7	1	4	0	2	0	2
20	4	11	2	7	1	4	0	2	0	2
21	3	7	2	5	1	4	0	3	0	2
22	3	9	2	10	1	5	0	4	0	5
23	3	7	2	4	1	3	0	3	0	3
24	3	7	2	9	1	4	0	3	0	3
25	3	7	2	9	1	4	0	3	0	3
26	3	7	2	9	1	4	0	3	0	3
27	3	7	2	9	1	4	0	3	0	3
28	4	9	3	8	2	6	0	3	0	3
29	5	9	4	8	2	6	1	2	1	3

No	Day 1		Day 2		Day 3		Day 4		Day 5	
	BC/SS/ PHMB	Bacti-gras®	BC/SS/ PHMB	Bacti-gras®	BC/SS PHMB	Bacti-gras®	BC/SS/ PHMB	Bacti-gras®	BC/SS/ PHMB	Bacti-gras®
30	5	9	4	8	2	6	1	2	1	3
31	5	9	4	8	2	6	1	2	1	3
32	5	9	4	8	2	6	1	2	1	3

Table 7I Biochemistry value of STSG donor site wound patients at day 0 and day 5 after operation

No	WBC[a] (4.5-11x10³/ul)		Neutrophil (1.8-7.8x10³/ul)		BUN[a] (7-20 mg/dl)		Cr[a] (0.5-1 mg/dl)		AST[a] (0-35 U/L)		ALT[a] (0-40 U/L)		ALP[a] (40-120 U/L)		Total Bilirubin (0.2-1.2)	
	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5
1	6.97	6.40	4.69	4.40	11.00	9.00	0.66	0.62	25.00	18.00	17.00	13.00	60.00	66.00	0.56	0.37
2	13.13	9.77	7.50	5.76	16.00	16.00	0.76	0.73	35.00	43.00	17.00	16.00	60.00	89.00	0.56	0.45
3	8.09	5.72	2.80	2.80	13.00	8.00	1.06	0.80	25.00	31.00	9.00	2.00	111.0	93.00	1.00	0.99
4	10.89	7.72	6.90	5.70	32.00	14.00	2.45	0.77	42.00	28.00	29.00	21.00	164.0	84.00	0.23	0.45
5	5.89	6.42	3.39	4.60	10.00	13.00	0.99	0.98	25.00	25.00	17.00	14.00	60.00	52.00	0.56	0.32
6	9.34	10.43	7.50	8.10	27.00	15.00	1.32	1.36	27.00	21.00	19.00	24.00	68.00	65.00	0.69	0.55
7	13.18	9.65	9.77	7.70	18.00	8.00	1.67	1.02	34.00	51.00	12.00	11.00	58.00	88.00	0.39	0.32
8	11.53	7.64	10.60	5.70	16.00	17.00	0.66	0.91	17.00	21.00	18.00	31.00	49.00	54.00	0.39	0.37
9	10.71	6.83	7.80	4.19	14.00	14.00	0.96	0.57	35.00	54.00	40.00	51.00	139.0	151.0	0.79	0.26
10	10.28	6.11	6.30	3.72	30.00	27.00	1.04	1.23	31.00	24.00	32.00	28.00	81.00	80.00	0.20	0.33
11	12.44	9.78	9.57	7.66	11.00	16.00	0.94	0.87	35.00	41.00	9.00	25.00	156.0	192.0	2.84	1.32

No	WBC[a] (4.5-11x10^3/ul)		Neutrophil (1.8-7.8x10^3/ul)		BUN[a] (7-20 mg/dl)		Cr[a] (0.5-1 mg/dl)		AST[a] (0-35 U/L)		ALT[a] (0-40 U/L)		ALP[a] (40-120 U/L)		Total Bilirubin (0.2-1.2)	
	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5	Day 0	Day 5
13	10.41	6.30	9.46	4.91	19.00	18.00	0.84	0.72	36.00	69.00	20.00	39.00	51.00	95.00	1.09	0.39
14	18.58	8.94	14.60	6.07	14.00	15.00	1.40	1.21	24.00	31.00	21.00	15.00	42.00	46.00	0.52	0.79
15	19.90	15.18	14.81	12.17	13.00	7.00	1.07	0.60	26.00	32.00	40.00	52.00	74.00	99.00	1.11	1.77
16	7.55	6.53	5.07	4.50	9.00	9.00	0.60	0.63	28.00	26.00	14.00	15.00	60.00	61.00	0.50	0.64
17	10.80	7.72	7.50	5.70	17.00	19.00	1.02	1.06	16.00	3.00	49.00	45.00	60.00	84.00	0.44	0.35
18	7.76	9.57	4.79	6.10	12.00	12.00	0.88	0.77	32.00	21.00	49.00	23.00	78.00	84.00	0.56	0.80
19	15.56	8.24	12.62	11.36	7.00	10.00	0.73	0.73	19.00	28.00	12.00	21.00	88.00	95.00	0.36	0.32
20	9.84	8.32	4.47	4.14	20.00	18.00	0.72	0.64	23.00	19.00	52.00	19.00	64.00	63.00	0.43	0.36
21	11.78	7.72	7.36	4.11	6.00	3.00	0.69	0.65	25.00	48.00	13.00	28.00	100.0	100.0	0.71	0.62

[a] WBC: White blood cells, BUN: Blood urea nitrogen, Cr: Creatinine, AST: aspartate aminotransferase, ALT: Alanine aminotransferase, ALP: Alkaline phosphatase

Appendix J

Ethic certification approval

AF 02-12

The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University
Institute Building 2, 4 Floor, Soi Chulalongkorn 62, Phyat hai Rd., Bangkok 10330, Thailand,
Tel: 0-2218-8147 Fax: 0-2218-8147 E-mail: eccu@chula.ac.th

COA No. 127/2014

Certificate of Approval

Study Title No.113.1/57 : **SAFETY OF BIOCELLULOSE WITH SILK SERICIN AND PHMB DRESSING IN HEALTHY VOLUNTEERS**

Principal Investigator : ASSOC.PROF.DR.PORNANONG ARAMWIT

Place of Proposed Study/Institution : Faculty of Pharmaceutical Sciences, Chulalongkorn University

The Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University, Thailand, has approved constituted in accordance with the International Conference on Harmonization – Good Clinical Practice (ICH-GCP) and/or Code of Conduct in Animal Use of NRCT version 2000.

Signature: Prida Tasanapradit Signature: Nuntaree Chaichanawongsaroj

(Associate Professor Prida Tasanapradit, M.D.) Chairman

(Assistant Professor Dr. Nuntaree Chaichanawongsaroj) Secretary

Date of Approval : 4 September 2014 Approval Expire date : 3 September 2015

The approval documents including

1) Research proposal
2) Patient/Participant Information Sheet and Informed Consent Form
3) Researcher
4) Questionnaire

Protocol No. 113.1/57
Date of Approval - 4 SEP 2014
Approval Expire Date - 3 SEP 2015

The approved investigator must comply with the following conditions:

1. *The research/project activities must end on the approval expired date of the Ethics Review Committee for Research Involving Human Research Subjects, Health Science Group, Chulalongkorn University (ECCU). In case the research/project is unable to complete within that date, the project extension can be applied one month prior to the ECCU approval expired date.*
2. *Strictly conduct the research/project activities as written in the proposal.*
3. *Using only the documents that bearing the ECCU's seal of approval with the subjects/volunteers (including subject information sheet, consent form, invitation letter for project/research participation (if available).*
4. *Report to the ECCU for any serious adverse events within 5 working days*
5. *Report to the ECCU for any change of the research/project activities prior to conduct the activities.*
6. *Final report (AF 03-12) and abstract is required for a one year (or less) research/project and report within 30 days after the completion of the research/project. For thesis, abstract is required and report within 30 days after the completion of the research/project.*
7. *Annual progress report is needed for a two- year (or more) research/project and submit the progress report before the expire date of certificate. After the completion of the research/project processes as No. 6.*

AF 01-12

คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย
อาคารสถาบัน 2 ชั้น 4 ซอยจุฬาลงกรณ์ 62 ถนนพญาไท เขตปทุมวัน กรุงเทพฯ 10330
โทรศัพท์: 0-2218-8147 โทรสาร: 0-2218-8147 E-mail: eccu@chula.ac.th

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COA No. 127/2557

ใบรับรองโครงการวิจัย

โครงการวิจัยที่ 113.1/57 : ความปลอดภัยของแผ่นปิดแผลไบโอเซลลูโลสผสมโปรตีนกาวไหม เซริซินและสารฆ่าเชื้อ PHMB ในอาสาสมัครสุขภาพดี

ผู้วิจัยหลัก : รองศาสตราจารย์ ดร.พรอนงค์ อร่ามวิทย์

หน่วยงาน : คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

คณะกรรมการพิจารณาจริยธรรมการวิจัยในคน กลุ่มสหสถาบัน ชุดที่ 1 จุฬาลงกรณ์มหาวิทยาลัย ได้พิจารณา โดยใช้หลัก ของ The International Conference on Harmonization – Good Clinical Practice (ICH-GCP) อนุมัติให้ดำเนินการศึกษาวิจัยเรื่องดังกล่าวได้

ลงนาม.....
(รองศาสตราจารย์ นายแพทย์ปรีดา ทัศนประดิษฐ)
ประธาน

ลงนาม.....
(ผู้ช่วยศาสตราจารย์ ดร.นันทรี ชัยชนะวงศ์โรจน์)
กรรมการและเลขานุการ

วันที่รับรอง : 4 กันยายน 2557 **วันหมดอายุ** : 3 กันยายน 2558

เอกสารที่คณะกรรมการรับรอง

1) โครงการวิจัย
2) ข้อมูลสำหรับกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัยและใบยินยอมของกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัย
3) ผู้วิจัย
4) แบบสอบถาม

เลขที่โครงการวิจัย 113.1/57
วันที่รับรอง - 4 ก.ย. 2557
วันหมดอายุ - 3 ก.ย. 2558

เงื่อนไข

1. ข้าพเจ้ารับทราบว่าเป็นการผิดจริยธรรม หากดำเนินการเก็บข้อมูลการวิจัยก่อนได้รับการอนุมัติจากคณะกรรมการพิจารณาจริยธรรมการวิจัยฯ
2. หากใบรับรองโครงการวิจัยหมดอายุ การดำเนินการวิจัยต้องยุติ เมื่อต้องการต่ออายุต้องขออนุมัติใหม่ล่วงหน้าไม่ต่ำกว่า 1 เดือน พร้อมส่งรายงานความก้าวหน้าการวิจัย
3. ต้องดำเนินการวิจัยตามที่ระบุไว้ในโครงการวิจัยอย่างเคร่งครัด
4. ใช้เอกสารข้อมูลสำหรับกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัย ใบยินยอมของกลุ่มประชากรหรือผู้มีส่วนร่วมในการวิจัย และเอกสารเชิญเข้าร่วมวิจัย (ถ้ามี) **เฉพาะที่ประทับตราคณะกรรมการเท่านั้น**
5. หากเกิดเหตุการณ์ไม่พึงประสงค์ร้ายแรงในสถานที่เก็บข้อมูลที่ขออนุมัติจากคณะกรรมการ ต้องรายงานคณะกรรมการภายใน 5 วันทำการ
6. หากมีการเปลี่ยนแปลงการดำเนินการวิจัย ให้ส่งคณะกรรมการพิจารณารับรองก่อนดำเนินการ
7. โครงการวิจัยไม่เกิน 1 ปี ส่งแบบรายงานสิ้นสุดโครงการวิจัย (AF 03-12) และบทคัดย่อผลการวิจัยภายใน 30 วัน เมื่อโครงการวิจัยเสร็จสิ้น สำหรับโครงการวิจัยที่เป็นวิทยานิพนธ์ให้ส่งบทคัดย่อผลการวิจัย ภายใน 30 วัน เมื่อโครงการวิจัยเสร็จสิ้น

COA No. 843/2015

IRB No. 242/58

INSTITUTIONAL REVIEW BOARD

Faculty of Medicine, Chulalongkorn University

1873 Rama 4 Road, Patumwan, Bangkok 10330, Thailand, Tel 662-256-4493

Certificate of Approval

The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, has approved the following study in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guideline and International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Study Title	: Clinical efficacy of the novel biocellulose wound dressing containing silk sericin and polyhexamethylene biguanide for split-thickness skin graft donor sites
Study Code	: -
Principal Investigator	: Miss Supamas Napavichayanun
Affiliation of PI	: Faculty of Pharmaceutical Science, Chulalongkorn University.
Review Method	: Full board
Continuing Report	: At least once annually or submit the final report if finished.

Document Reviewed :

1. Research Proposal Version 5 Date 23 November 2015
2. Protocol Synopsis Version 5 Date 23 November 2015
3. Information sheet for research participant Version 4 Dated 23 November 2015
4. Informed Consent Form Version 3 Dated 2 November 2015
5. Curriculum Vitae
6. GCP Training
7. Appendix A : Demographic data Version 3 Date 23 November 2015
8. Appendix B : Follow up report Version 3 Date 23 November 2015

9. Appendix C : Adverse event report Version 3 Date 23 November 2015
10. Appendix D : Vancouver scar scale report Version 3 Date 23 November 2015
11. Questionnaire Version 2 Date 2 November 2015
12. Budgets Version 3 Date 2 November 2015

Signature:..... Signature:.....

(Emeritus Professor Tada Sueblinvong MD) (Assistant Professor Prapapan Rajatapiti MD, PhD)

Chairperson Member and Secretary

The Institutional Review Board Secretary The Institutional Review Board

Date of Approval : December 3, 2015

Approval Expire Date : December 2, 2016

Approval granted is subject to the following conditions: (see back of this Certificate)

All approved investigators must comply with the following conditions:

1. Strictly conduct the research as required by the protocol;
2. Use only the information sheet, consent form (and recruitment materials, if any), interview outlines and/or questionnaires bearing the Institutional Review Board's seal of approval ; and return one copy of such documents of the first subject recruited to the Institutional Review Board (IRB) for the record;
3. Report to the Institutional Review Board any serious adverse event or any changes in the research activity within five working days;
4. Provide reports to the Institutional Review Board concerning the progress of the research upon the specified period of time or when requested;
5. If the study cannot be finished within the expire date of the approval certificate, the investigator is obliged to reapply for approval at least one month before the date of expiration.
6. If the research project is completed, the researcher must be form the Faculty of Medicine, Chulalongkorn University.

* A list of the Institutional Review Board members (names and positions) present at the meeting of Institutional Review Board on the date of approval of this study has been attached. All approved documents will be forwarded to the principal investigator.

COA No. 843/2015

IRB No. 242/58

คณะกรรมการพิจารณาจริยธรรมการวิจัย
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
1873 ถ.พระราม 4 เขตปทุมวัน กรุงเทพฯ 10330 โทร. 0-2256-4493

เอกสารรับรองโครงการวิจัย

คณะกรรมการจริยธรรมการวิจัยในคน คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ดำเนินการให้การรับรองโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากลได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการ	: ประสิทธิศักย์ทางคลินิกของแผ่นปิดแผลไบโอเซลลูโลสผสมโปรตีนกาวไหมแซริซินและสารฆ่าเชื้อ โพลีเฮกซะเมธิลีนไบกัวไนด์ในการรักษาบาดแผลที่ถูกตัดผิวหนังบางส่วนเพื่อการปลูกถ่าย
เลขที่โครงการวิจัย	: -
ผู้วิจัยหลัก	: นางสาวศุภมาส นภาวิชยานันท์
สังกัดหน่วยงาน	: คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
วิธีทบทวน	: คณะกรรมการเต็มชุด
รายงานความก้าวหน้า	: ส่งรายงานความก้าวหน้าอย่างน้อย 1 ครั้ง/ปี หรือส่งรายงานฉบับสมบูรณ์หากดำเนินโครงการเสร็จสิ้นก่อน 1 ปี

เอกสารรับรอง :

1. Research Proposal Version 5 Date 23 November 2015
2. โครงการวิจัยฉบับย่อ Version 5 Date 23 November 2015
3. เอกสารข้อมูลคำอธิบายสำหรับผู้เข้าร่วมในโครงการวิจัย Version 4 Dated 23 November 2015
4. เอกสารแสดงความยินยอมเข้าร่วมในโครงการวิจัย Version 3 Dated 2 November 2015
5. ประวัติผู้วิจัย
6. GCP Training

7. Appendix A : Demographic data Version 3 Date 23 November 2015
8. Appendix B : Follow up report Version 3 Date 23 November 2015
9. Appendix C : Adverse event report Version 3 Date 23 November 2015
10. Appendix D : Vancouver scar scale report Version 3 Date 23 November 2015
11. แบบสอบถาม Version 2 Date 2 November 2015
12. Budgets Version 3 Date 2 November 2015

ลงนาม ..... ลงนาม.....

(ศาสตราจารย์กิตติคุณแพทย์หญิงธาดา สิบหลินวงศ์)
ประธาน
คณะกรรมการพิจารณาจริยธรรมการวิจัย

(ผู้ช่วยศาสตราจารย์ ดร.พญ.ประภาพรรณ รัชตะปิติ)
กรรมการและเลขานุการ
คณะกรรมการพิจารณาจริยธรรมการวิจัย

วันที่รับรอง : 3 ธันวาคม 2558
วันหมดอายุ : 2 ธันวาคม 2559

ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

นักวิจัยทุกท่านที่ผ่านการรับรองจริยธรรมการวิจัยต้องปฏิบัติดังต่อไปนี้

1. ดำเนินการวิจัยตามที่ระบุไว้ในโครงร่างการวิจัยอย่างเคร่งครัด
2. ใช้เอกสารแนะนำอาสาสมัคร ใบยินยอม (และเอกสารเชิญเข้าร่วมวิจัยหรือใบโฆษณาถ้ามี) แบบสัมภาษณ์ และหรือ แบบสอบถาม เฉพาะที่มีตราประทับของคณะกรรมการพิจารณาจริยธรรมเท่านั้น และส่งสำเนาเอกสารดังกล่าวที่ใช้กับผู้เข้าร่วมวิจัยจริงรายแรกมาที่ฝ่ายวิจัย คณะแพทยศาสตร์ เพื่อเก็บไว้เป็นหลักฐาน
3. รายงานเหตุการณ์ไม่พึงประสงค์ร้ายแรงที่เกิดขึ้นหรือการเปลี่ยนแปลงกิจกรรมวิจัยใดๆ ต่อคณะกรรมการพิจารณาจริยธรรมการวิจัย ภายใน 5 วันทำการ
4. ส่งรายงานความก้าวหน้าต่อคณะกรรมการพิจารณาจริยธรรมการวิจัย ตามเวลาที่กำหนดหรือเมื่อได้รับการร้องขอ
5. หากการวิจัยไม่สามารถดำเนินการเสร็จสิ้นภายในกำหนด ผู้วิจัยต้องยื่นขออนุมัติใหม่ก่อนอย่างน้อย 1 เดือน
6. หากการวิจัยเสร็จสมบูรณ์ผู้วิจัยต้องแจ้งปิดโครงการตามแบบฟอร์มของคณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

* รายชื่อของคณะกรรมการจริยธรรมการวิจัยในคน (ชื่อและตำแหน่ง) ที่อยู่ในที่ประชุมวันที่รับรองโครงการวิจัยได้แนบมาด้วย เอกสารที่รับรองทั้งหมดจะถูกส่งไปยังผู้วิจัยหลัก

IRB. No. 242/58

INSTITUTIONAL REVIEW BOARD

Faculty of Medicine, Chulalongkorn University

1873 Rama IV Road, Patumwan, Bangkok 10330, Thailand, Tel 662-256-4493

Approval of Documents related to Study Protocol

The Institutional Review Board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand, has approved/acknowledged the following study which is to be carried out in compliance with the International guidelines for human research protection as Declaration of Helsinki, The Belmont Report, CIOMS Guidelines and International Conference on Harmonization in Good Clinical Practice (ICH-GCP)

Study Title : CLINICAL EFFICACY OF BIOCELLULOSE WOUND DRESSING CONTAINING SILK SERICIN AND POLYHEXAMETHYLENE BIGUANIDE FOR SPLIT-THICKNESS SKIN GRAFT DONOR SITES

Study Code : -

Principal Investigator : Miss Supamas Napavichayanun

Affiliation of PI : Faculty of Pharmaceutical Science, Chulalongkorn University

Document Reviewed :

1. Change Protocol title (Thai and English)

Signature ..... Signature .....

(Assistant Professor Apichai Vasuratna MD)
Vice-Chairman, Acting Chairman
The Institutional Review Board

(Napakkawat Buathong, PhD)
Member and Assistant Secretary, Acting
Secretary The Institutional Review Board

Date of Approval : November 30, 2017

Approval granted is subject to the following conditions: (see back of this Certificate)

All approved investigators must comply with the following conditions:

1. Strictly conduct the research as required by the protocol;
2. Use only the information sheet, consent form (and recruitment materials, if any), interview outlines and/or questionnaires bearing the Institutional Review Board's seal of approval ; and return one copy of such documents of the first subject recruited to the Institutional Review Board (IRB) for record keeping;
3. Report to the Institutional Review Board any serious adverse event or any changes in the research activity within five working days;
4. Provide reports to the Institutional Review Board concerning the progress of the research upon the specified period of time or when requested;
5. If the study cannot be finished within the expiring date of the approval on the certificate, the investigator is obliged to reapply for approval at least one month before the date of expiration.
6. All the above approved documents expire on the same date of the previously approved protocol (Protocol Number.....040158.....)

IRB. No. 242/58

คณะกรรมการพิจารณาจริยธรรมการวิจัย
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
1873 ถนนพระราม 4 เขตปทุมวัน กรุงเทพฯ 10330 โทร 02-256-4493

เอกสารรับรองโครงการวิจัยส่วนเพิ่มเติม

คณะกรรมการพิจารณาจริยธรรมการวิจัย คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ดำเนินการให้การรับรอง/รับทราบ เอกสารที่เกี่ยวข้องกับโครงการวิจัยตามแนวทางหลักจริยธรรมการวิจัยในคนที่เป็นมาตรฐานสากลได้แก่ Declaration of Helsinki, The Belmont Report, CIOMS Guideline และ International Conference on Harmonization in Good Clinical Practice หรือ ICH-GCP

ชื่อโครงการ : ประสิทธิศักย์ทางคลินิกของสิ่งตกแต่งแผลไบโอเซลลูโลสผสมเซริซินจากไหมและโพลีเฮกซะเมธิลีน ไบกัวไนด์ในการรักษาบาดแผลที่ถูกตัดผิวหนังบางส่วนเพื่อปลูกถ่าย

เลขที่โครงการวิจัย :

ผู้วิจัยหลัก : นางสาว ศุภมาส นภาวิชยานันท์

สังกัดหน่วยงาน : คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

เอกสารรับรอง :
1. เปลี่ยนชื่อเรื่องงานวิจัย

ลงนาม .....
(ผู้ช่วยศาสตราจารย์นายแพทย์อภิชัย วสุรัตน์)
รองประธานปฏิบัติหน้าที่แทนประธาน
คณะกรรมการพิจารณาจริยธรรมการวิจัย

ลงนาม.....
(ดร.ณภัควรรต บัวทอง)
กรรมการและผู้ช่วยเลขานุการฯ ปฏิบัติหน้าที่แทน
เลขานุการฯ คณะกรรมการพิจารณาจริยธรรมการวิจัย

วันที่รับรอง : 30 พฤศจิกายน 2560

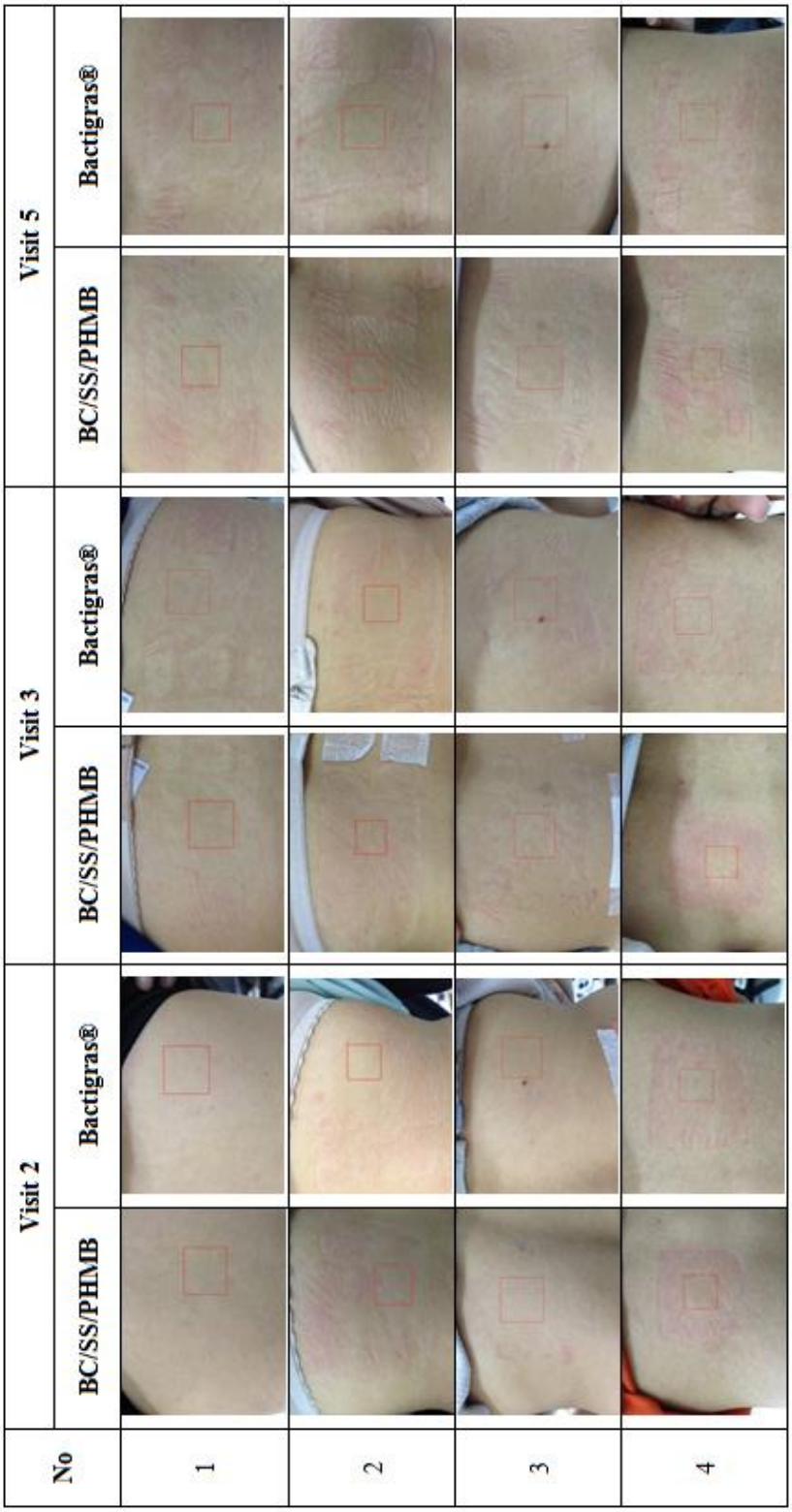
ทั้งนี้ การรับรองนี้มีเงื่อนไขดังที่ระบุไว้ด้านหลังทุกข้อ (ดูด้านหลังของเอกสารรับรองโครงการวิจัย)

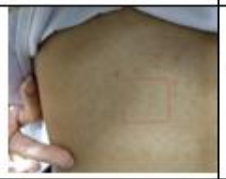
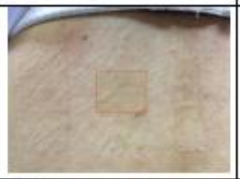
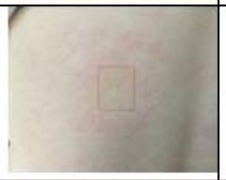
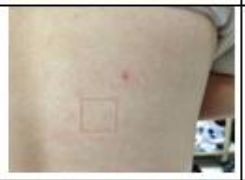
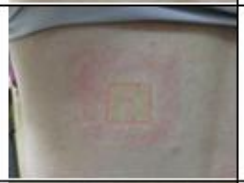
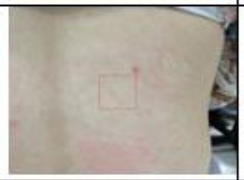
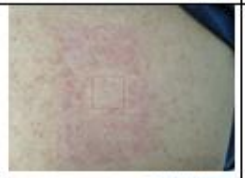
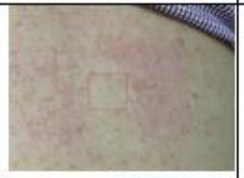
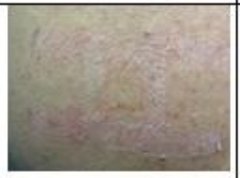
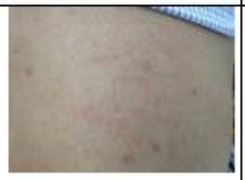
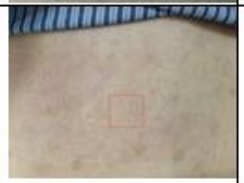
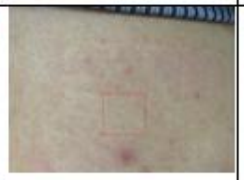
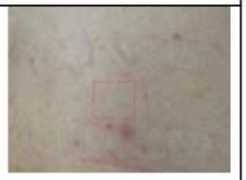
นักวิจัยทุกท่านที่ผ่านการรับรองจริยธรรมการวิจัยต้องปฏิบัติดังต่อไปนี้

1. ดำเนินการวิจัยตามที่ระบุไว้ในโครงร่างการวิจัยอย่างเคร่งครัด
2. ใช้เอกสารแนะนำอาสาสมัคร ใบยินยอม (และเอกสารเชิญเข้าร่วมวิจัยหรือใบโฆษณาถ้ามี) แบบสัมภาษณ์ และหรือ แบบสอบถาม เฉพาะที่มีตราประทับของคณะกรรมการพิจารณาจริยธรรมเท่านั้น และส่งสำเนาเอกสารดังกล่าวที่ใช้กับผู้เข้าร่วมวิจัยจริงรายแรกมาที่ฝ่ายวิจัย คณะแพทยศาสตร์ เพื่อเก็บไว้เป็นหลักฐาน
3. รายงานเหตุการณ์ไม่พึงประสงค์ร้ายแรงที่เกิดขึ้นหรือการเปลี่ยนแปลงกิจกรรมวิจัยใดๆ ต่อคณะกรรมการพิจารณาจริยธรรมการวิจัย ภายใน 5 วันทำการ
4. ส่งรายงานความก้าวหน้าต่อคณะกรรมการพิจารณาจริยธรรมการวิจัย ตามเวลาที่กำหนดหรือเมื่อได้รับการร้องขอ
5. หากการวิจัยไม่สามารถดำเนินการเสร็จสิ้นภายในกำหนด ผู้วิจัยต้องยื่นขออนุมัติใหม่ก่อน อย่างน้อย 1 เดือน
6. เอกสารทุกฉบับที่ได้รับการรับรองครั้งนี้ หมดอายุตามอายุของโครงร่างการวิจัยที่ได้รับการรับรองก่อนหน้านี้ (หมายเลขโครงการ 242/58)

Appendix K

Skin picture of healthy volunteers after covered with dressings



No	Visit 2		Visit 3		Visit 5	
	BC/SS/PHMB	Bactigras®	BC/SS/PHMB	Bactigras®	BC/SS/PHMB	Bactigras®
5						
6						
7						
8						

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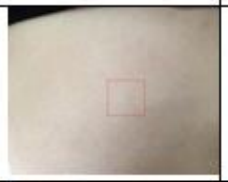
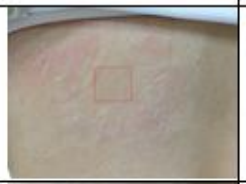
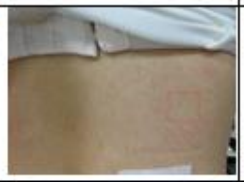
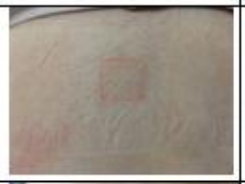
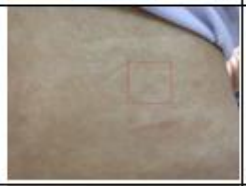
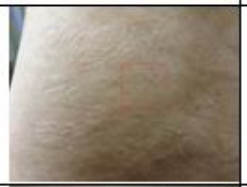
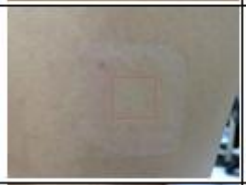
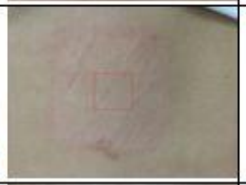
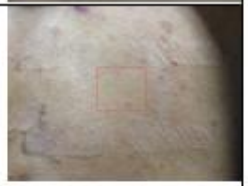
9						
10						
11						
12						

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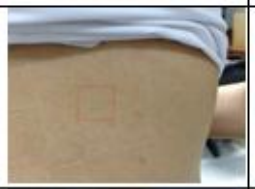
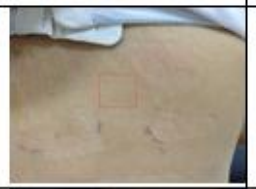
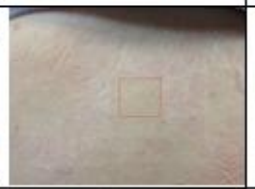
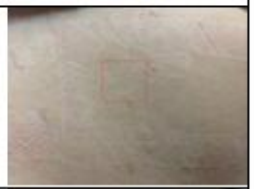
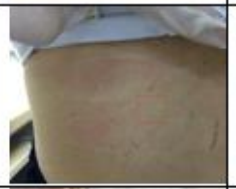
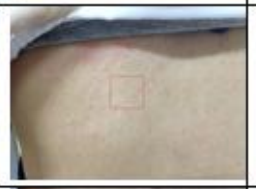
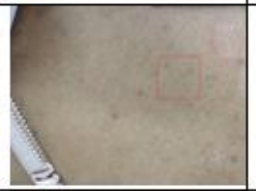
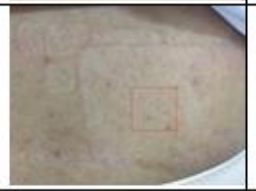
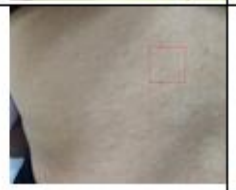
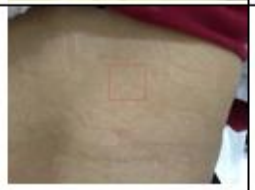
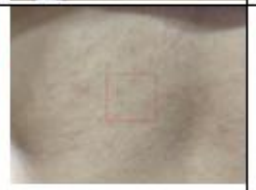
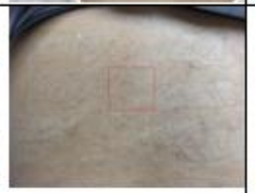
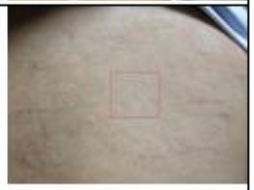
13						
14						
15						
16						

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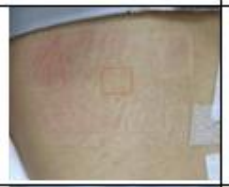
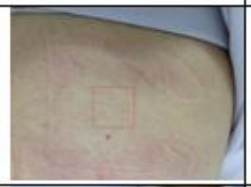
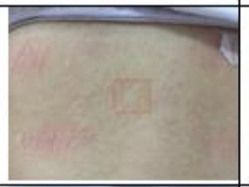
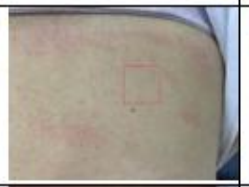
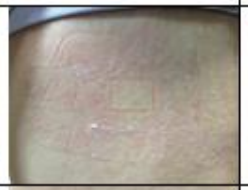
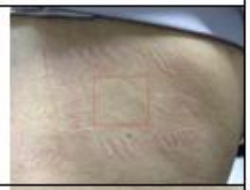
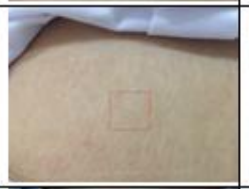
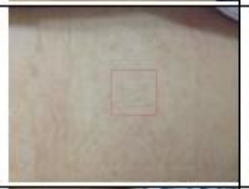
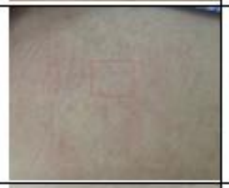
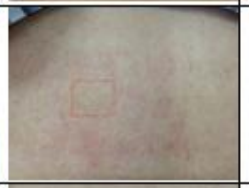
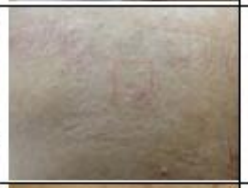
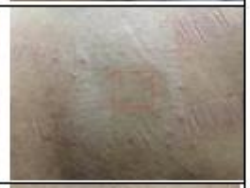
17						
18						
19						
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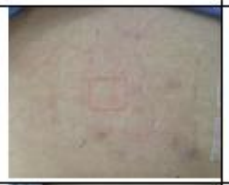
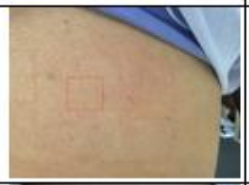
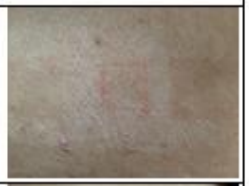
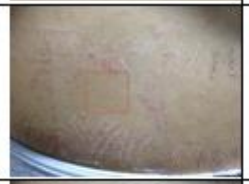
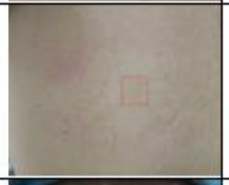
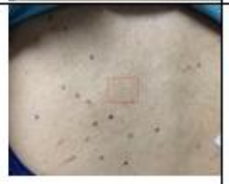
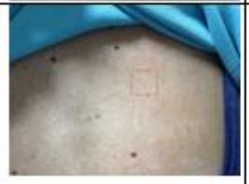
21						
22						
23						
24						

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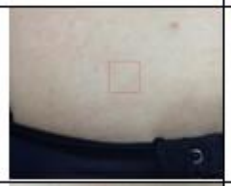
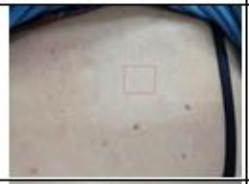
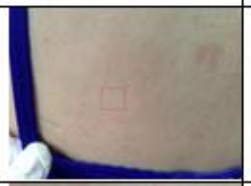
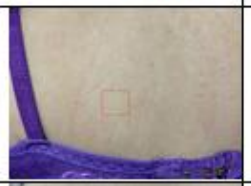
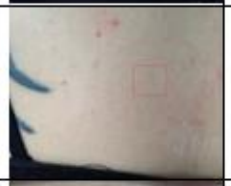
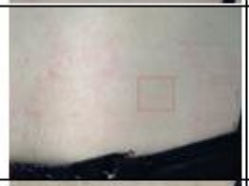
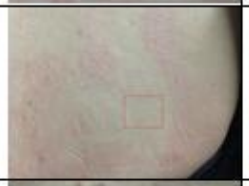
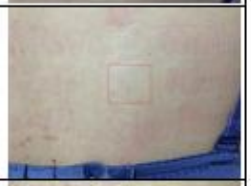
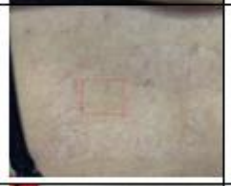
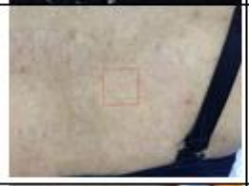
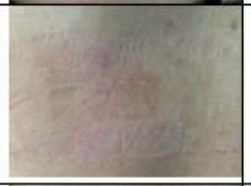
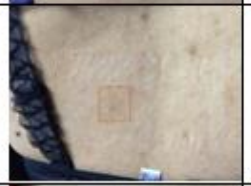
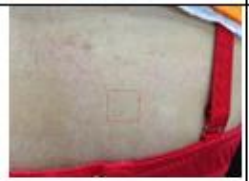
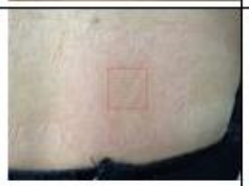
25						
26						
27						
28						

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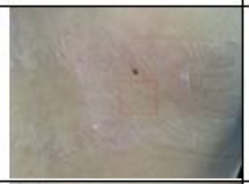
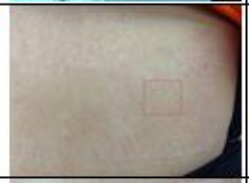
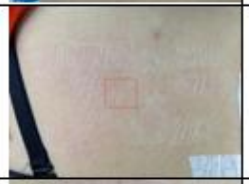
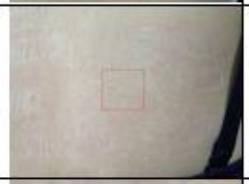
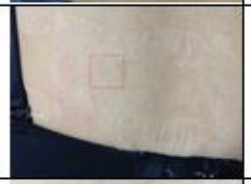
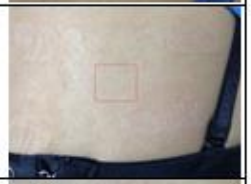
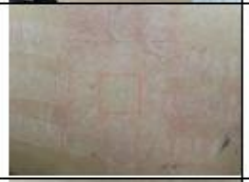
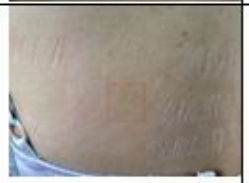
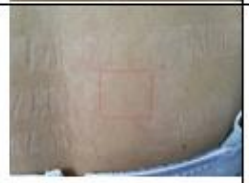
29						
30						
31						
32						

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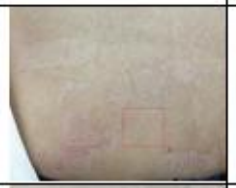
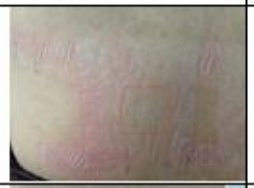
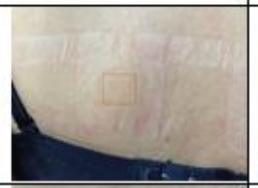
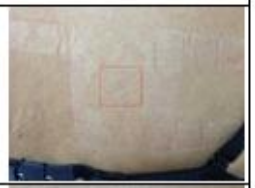
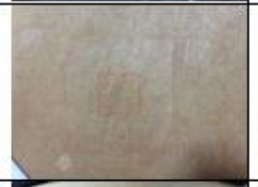
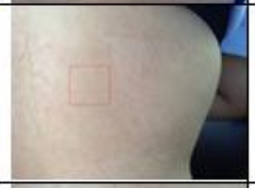
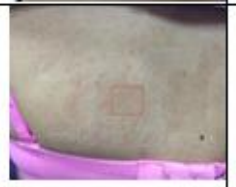
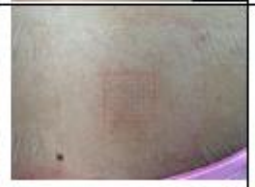
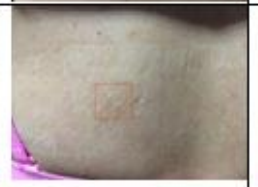
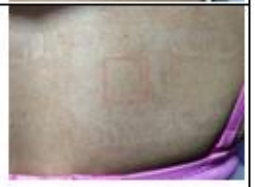
33						
34						
35						
36						

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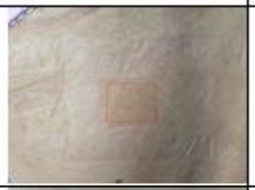
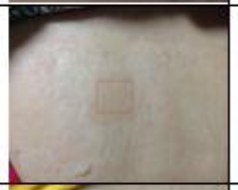
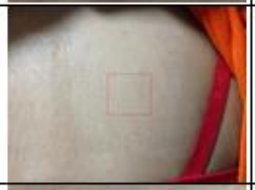
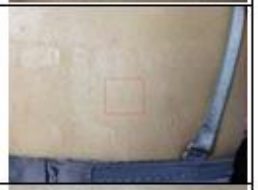
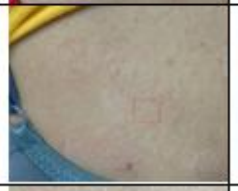
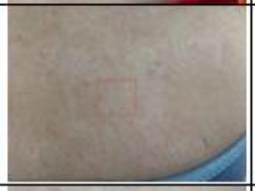
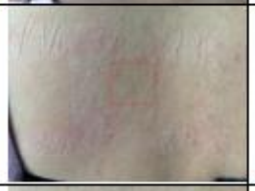
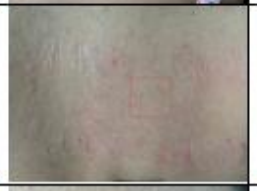
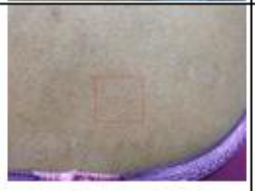
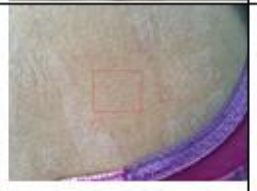
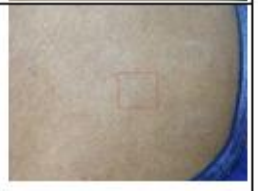
37						
38						
39						
40						

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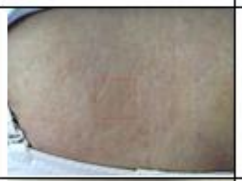
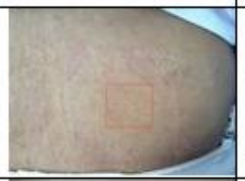
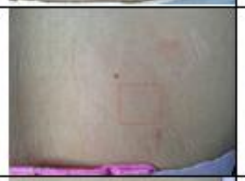
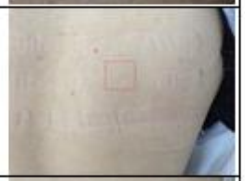
41						
42						
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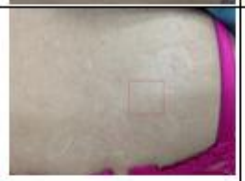
44						

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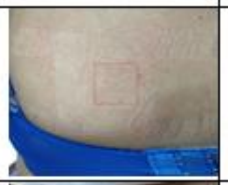
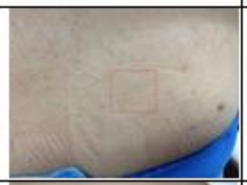
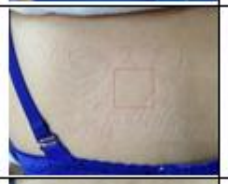
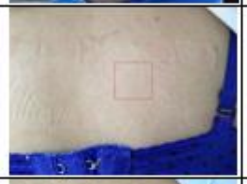
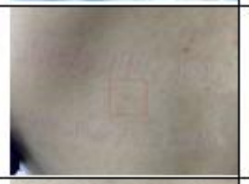
45						
46						
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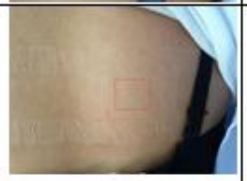
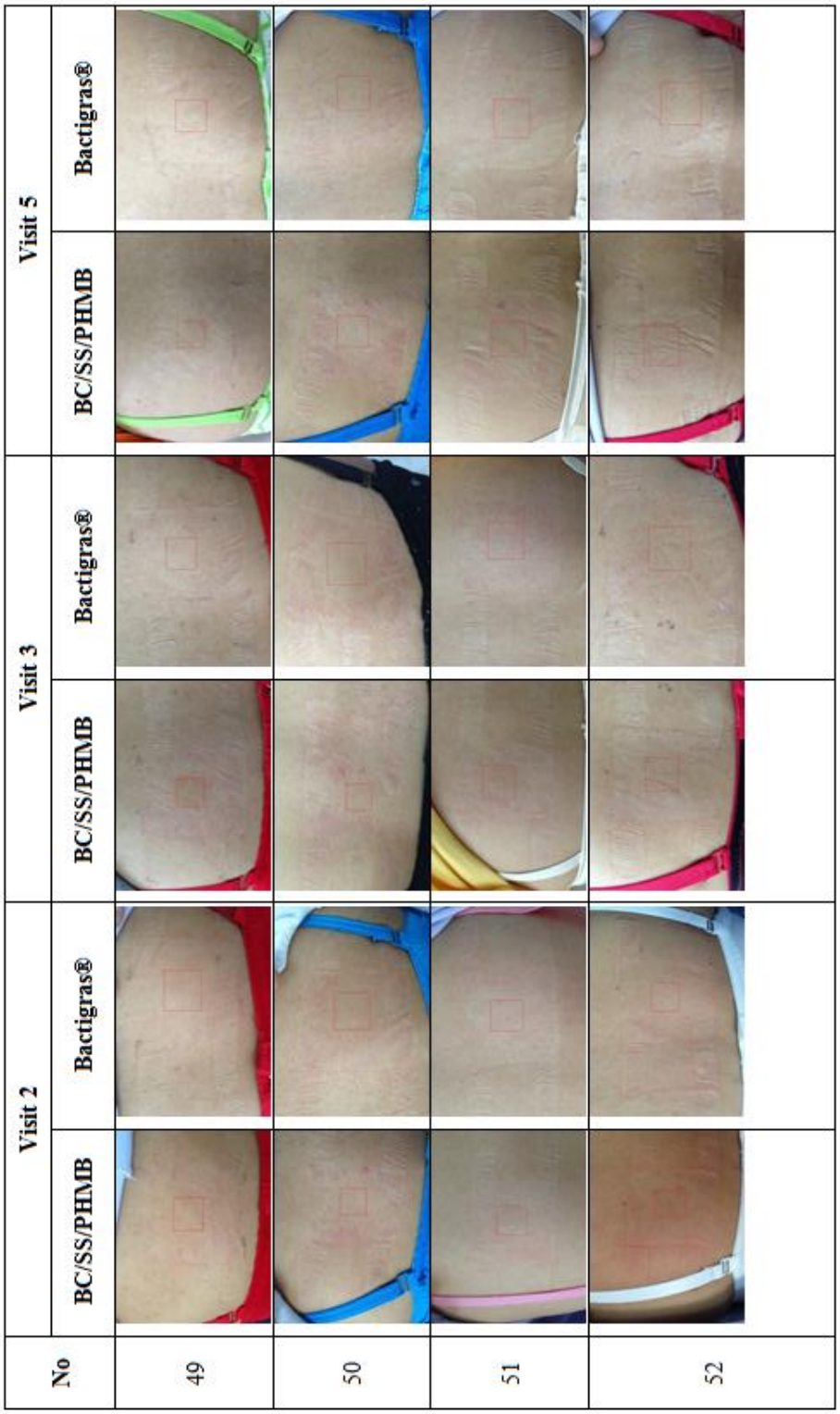
48						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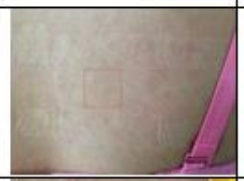
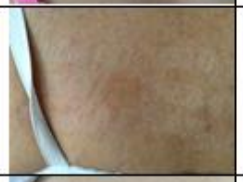
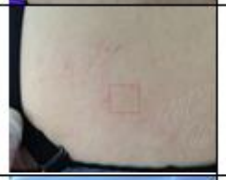
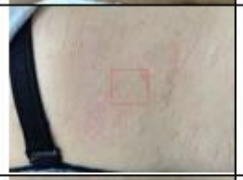
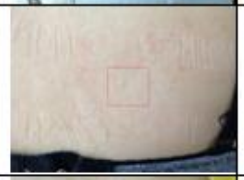
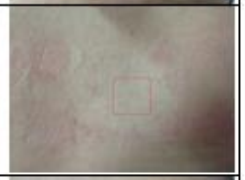
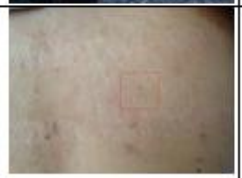
53						
54						
55						
56						

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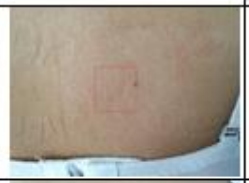
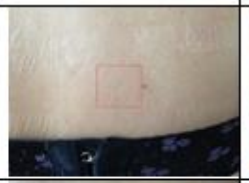
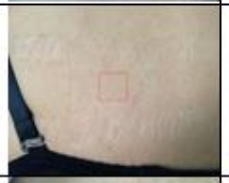
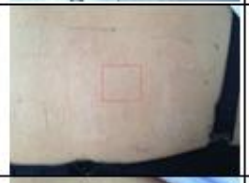
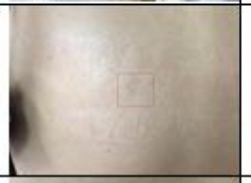
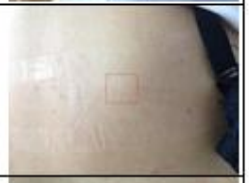
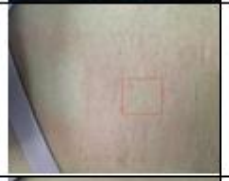
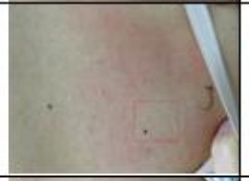
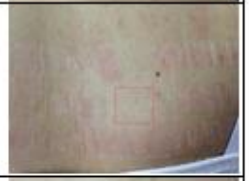
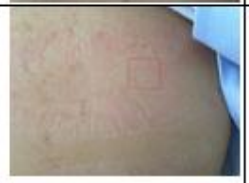
57						
58						
59						
60						

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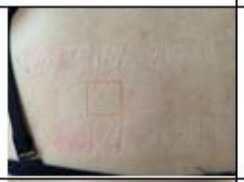
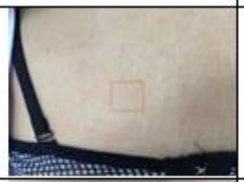
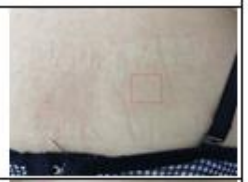
61						
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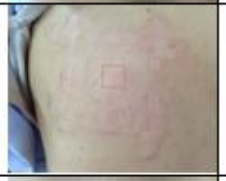
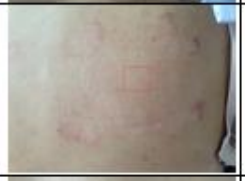
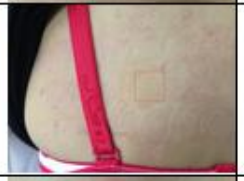
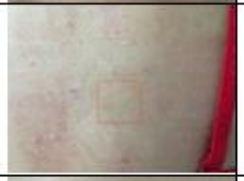
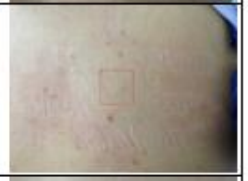
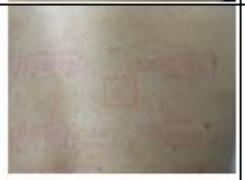
63						
64						

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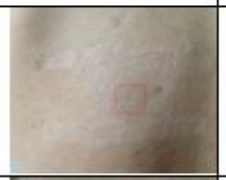
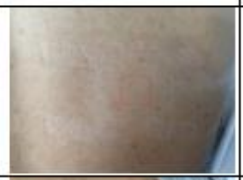
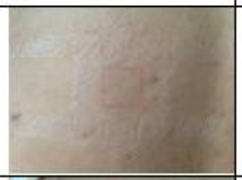
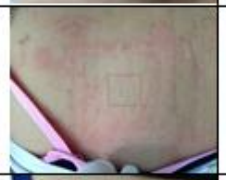
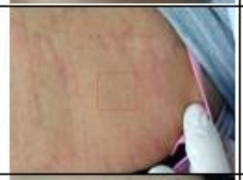
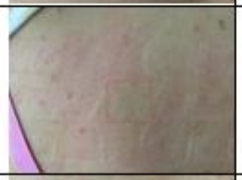
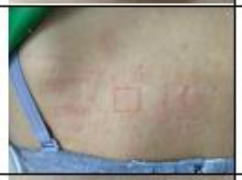
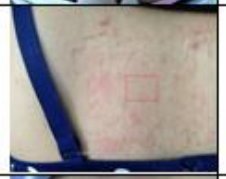
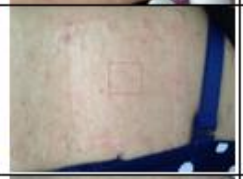
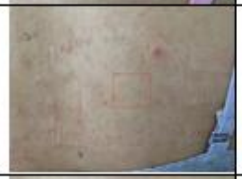
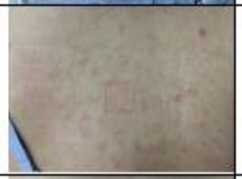
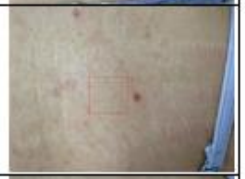
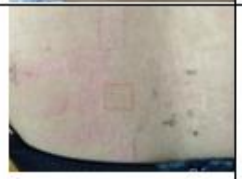
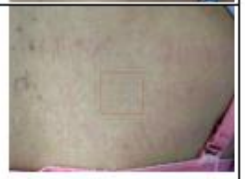
65						
66						
67						
68						

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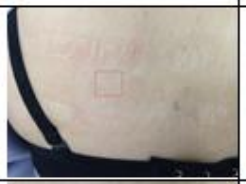
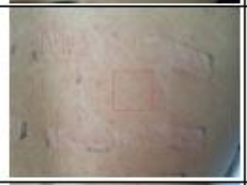
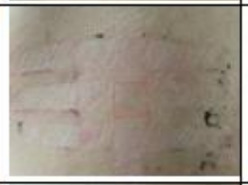
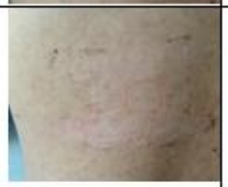
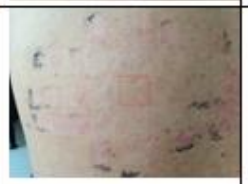
69						
70						
71						
72						

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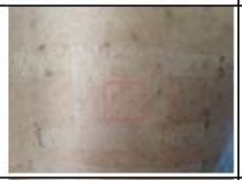
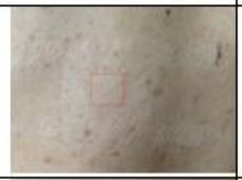
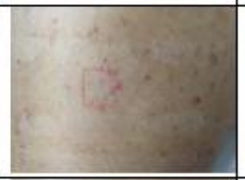
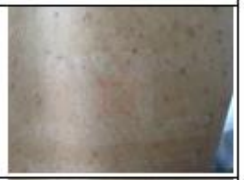
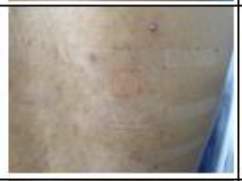
73						
74						
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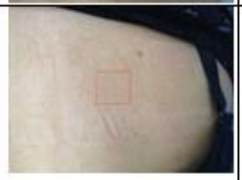
76						

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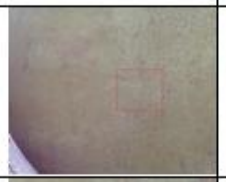
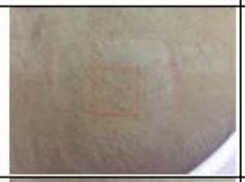
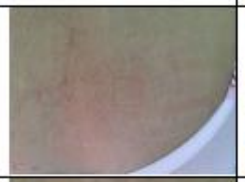
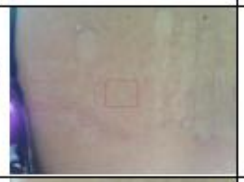
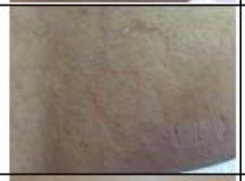
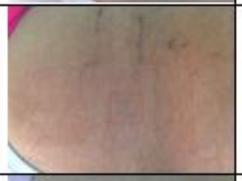
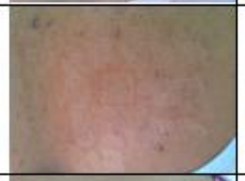
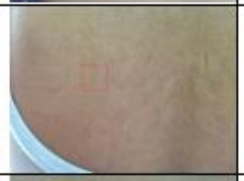
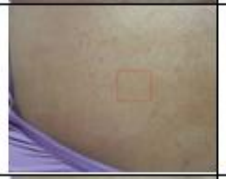
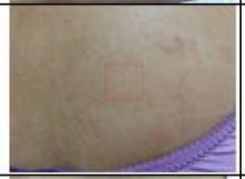
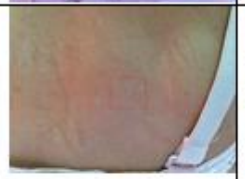
77						
78						
79						
80						

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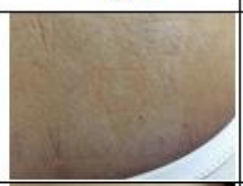
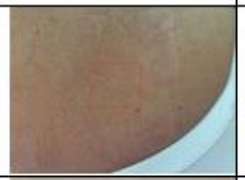
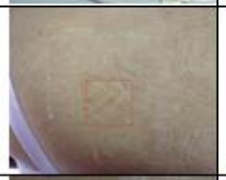
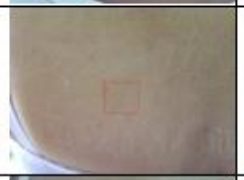
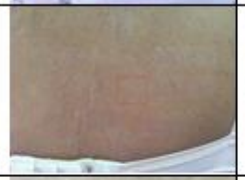
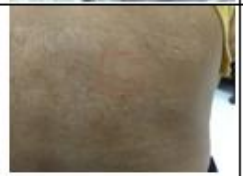
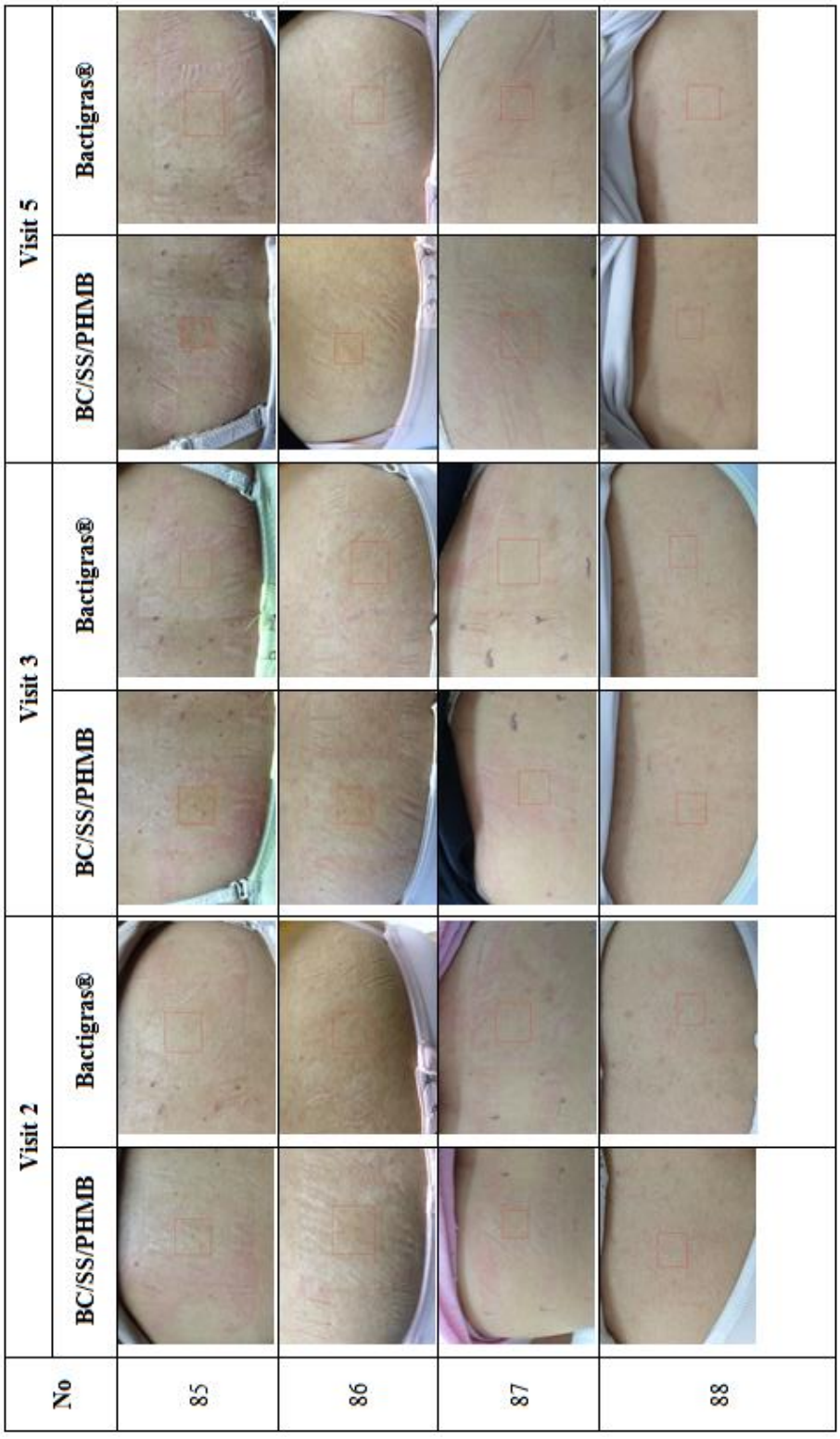
81						
82						
83						
84						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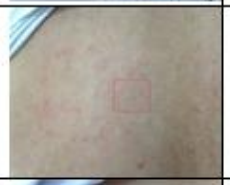
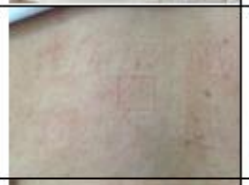
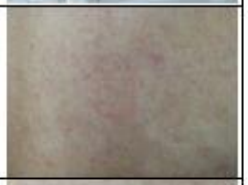
90						
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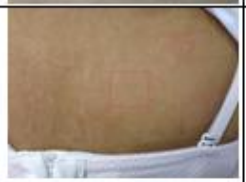
92						

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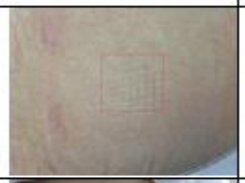
93						
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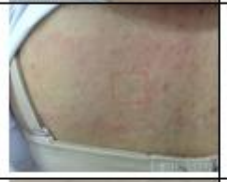
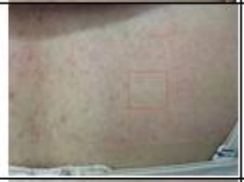
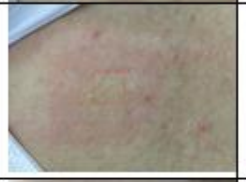
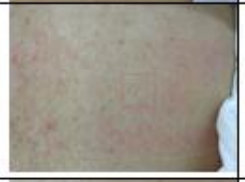
95						
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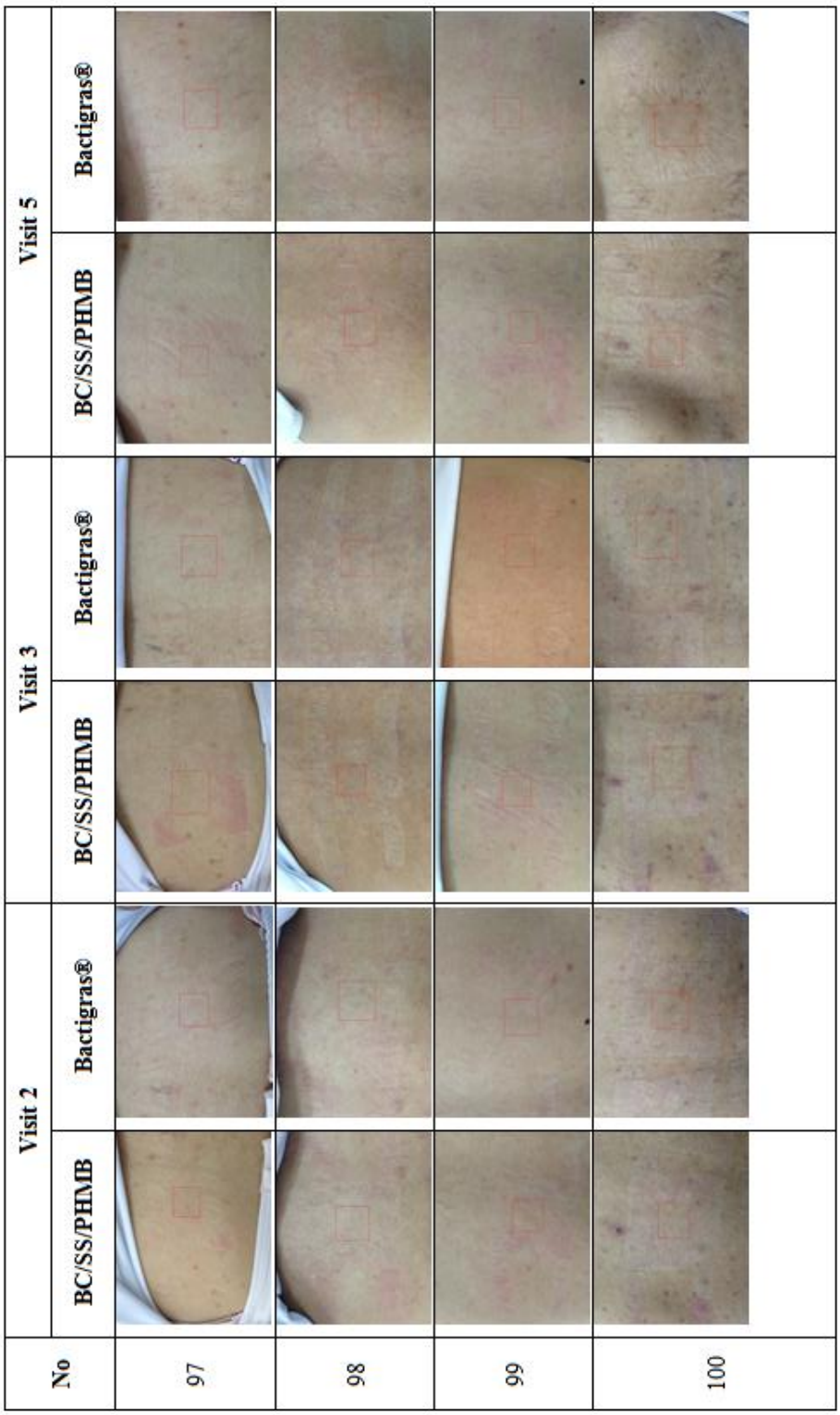
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Visit 3
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Visit 5
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Bactigras®
97
98
99
100

No	Visit 2		Visit 3		Visit 5	
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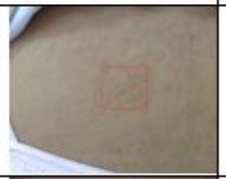
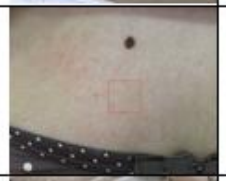
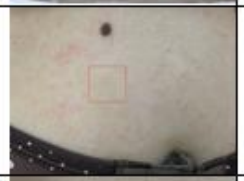
101						
102						
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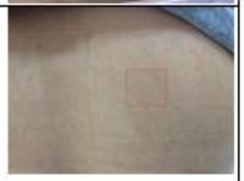
104						

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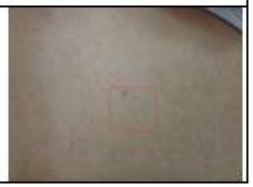
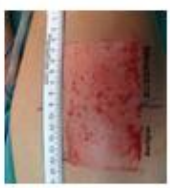
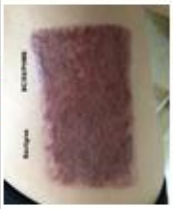
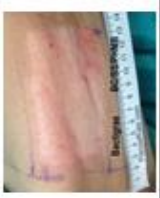
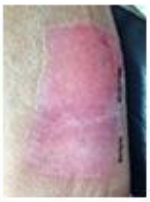
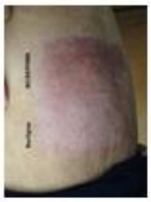
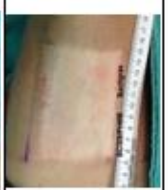
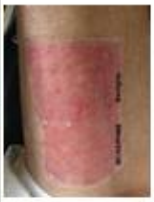
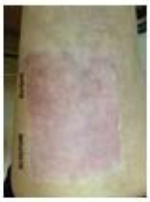
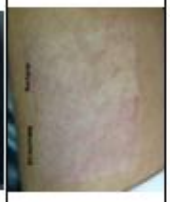
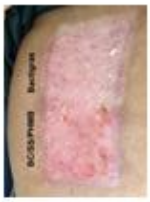
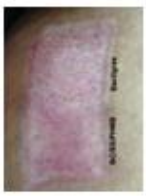
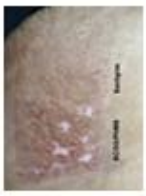
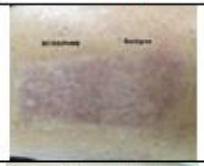
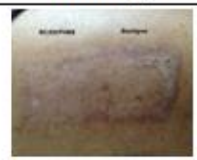
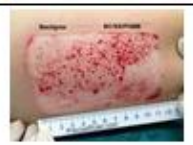
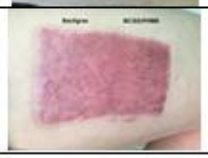
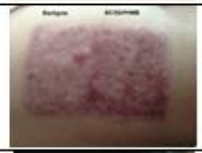
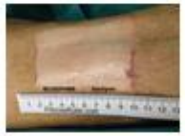
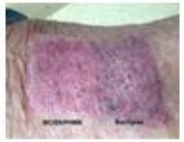
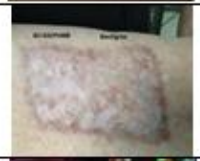
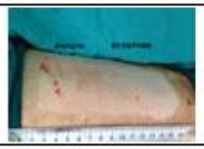
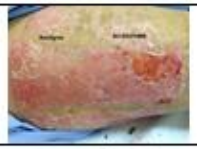
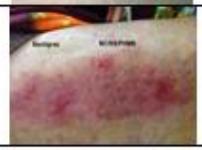
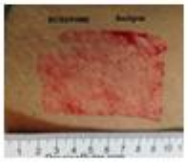
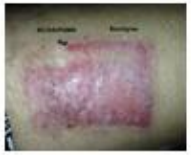
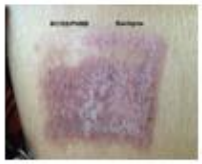
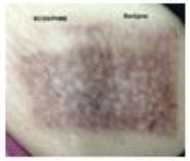
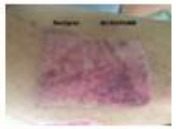
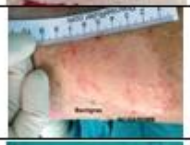
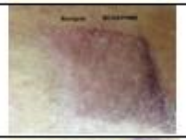
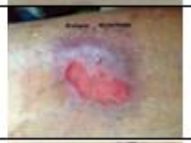
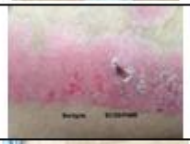
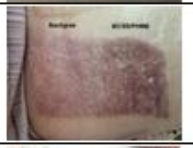
105						

Appendix L

STSG donor site wound picture after covered with dressings

No	Day 0	Healing time	1 month after wound healing	3 months after wound healing	6 months after wound healing
01					
02					
03					
04					

No	Day 0	Healing time	1 month after wound healing	3 months after wound healing	6 months after wound healing
05					
06					
07					
08					
09					

No	Day 0	Healing time	1 month after wound healing	3 months after wound healing	6 months after wound healing
10					
11					
12					
13					
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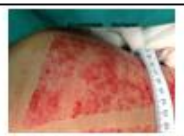
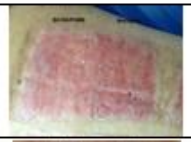
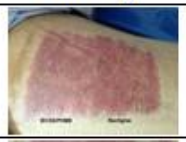
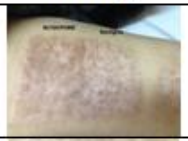
No	Day 0	Healing time	1 month after wound healing	3 months after wound healing	6 months after wound healing
16					
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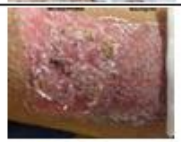
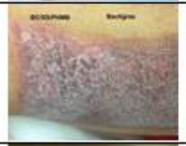
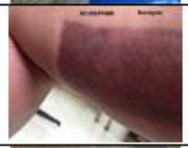
18					
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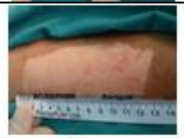
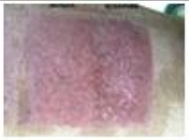
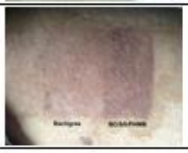
20					
21					

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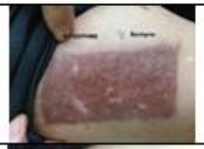
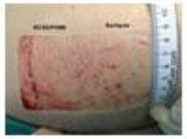
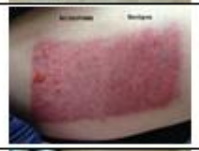
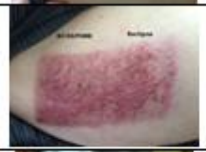
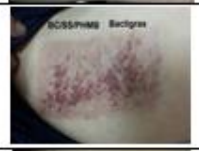
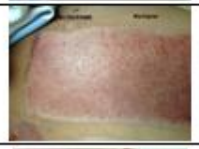
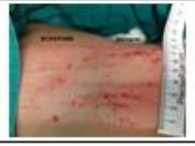
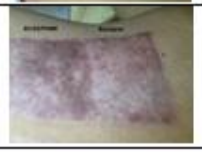
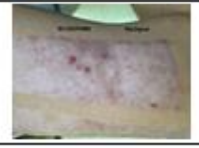
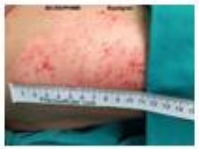
22					
23					
24					
25					
26					

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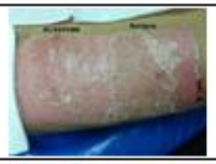
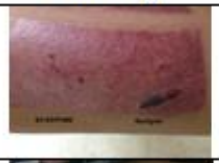
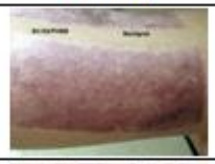
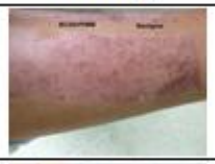
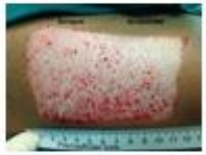
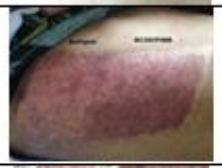
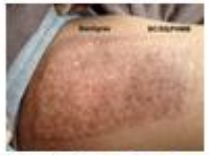
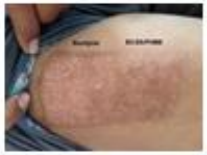
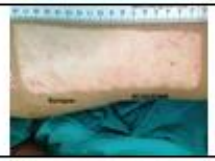
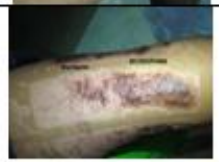
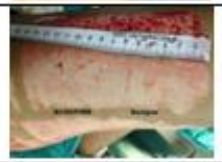
27					
28					
29					
30					
31					
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Appendix M

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Interaction and effectiveness of antimicrobials along with healing-promoting agents in a novel biocellulose wound dressing

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ABSTRACT

An ideal wound dressing should keep the wound moist, allow oxygen permeation, adsorb wound exudate, accelerate re-epithelialization for wound closure, reduce pain and healing time, and prevent infection. Our novel biocellulose-based wound dressing was composed of three components: 1) biocellulose (BC), intended to create a moist and oxygen-permeated environment with exudate adsorption; 2) silk sericin (SS) known for its enhancement of collagen type I production, which is critical for re-epithelialization; and 3) the antiseptic polyhexamethylene biguanide (PHMB). To deliver an effective BC wound dressing, the interactions between the components (PHMB vs. SS) needed to be thoroughly analyzed. In this study, we investigated important parameters such as the loading sequence, loading concentration, and loading amount of the active compounds to ensure that the BC wound dressing could provide both antimicrobial activity and promote collagen production during healing. The loading sequence of SS and PHMB into BC was critical to maintain PHMB antimicrobial activity; silk sericin needed to be loaded before PHMB to avoid any negative impacts. The minimum PHMB concentration was 0.3% w/v for effective elimination of all tested bacteria (*Bacillus subtilis*, *Staphylococcus aureus*, methicillin-resistant *S. aureus*, *Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*). The amounts of SS and PHMB in BC were optimized to ensure that the dressings released the optimal amounts of both SS to enhance fibroblast collagen production and PHMB for effective antimicrobial activity.

1. Introduction

The ideal wound dressing should promote a suitable environment for wound healing, enable fast tissue regeneration, reduce pain, and prevent infection during the healing process [1]. To accelerate the healing, the wound needs to be moisturized while the excess exudate and toxins must be removed to minimize maceration. In addition, oxygen must be able to permeate through the dressing to supply regenerating cells. Recently, biocellulose (also known as bacterial cellulose) has been developed for several applications, including wound dressing [2,3]. Unlike plant-based cellulose, biocellulose (BC) has an ultrafine network with high porosity which allows a high capability for water uptake [2,4], making it becomes an ideal material for the healing of high exudate wound. The highly uniaxial-oriented nanofibers (3–8 nm) of BC contribute to a high crystallinity content (60–80%) [2], providing impressive mechanical strength for ease of handling in the wet state. Furthermore, BC is considered as an electrostatically neutral material [5], enabling it appropriate for the loading of either positively-charged or negatively-charged bioactive compounds. In addition, the high water retention characteristics of BC create a moist wound healing environment [2,4], which allows for faster healing than a dried environment [6–8].

Up to date, commercial BC wound dressings are available. Some of them are incorporated with drugs or active compounds (i.e., iodine, chlorhexidine, and silver) for antimicrobial purpose. In this study, polyhexamethylene biguanide (PHMB) was chosen as an antiseptic drug to be incorporated in the BC dressing because it displays advantages over other antiseptics [1,9,10]. PHMB (molecular weight 3000 Da) is known as a cationic and strong base which interacts with acids and negatively charged molecules, such as phospholipids found in bacterial membranes [11]. The antimicrobial activity is based on the interaction between PHMB and bacterial phospholipids, which disrupts the integrity of the bacterial membrane, leading to the intrusion of PHMB into the cytoplasm, causing a malfunction in metabolic activity and resulting in the death of the bacterium [12,13]. It is reported that PHMB shows antimicrobial effect against various microorganisms such as yeasts, fungi, and bacteria (both Gram-positive and Gram-negative) with low toxicity in mammalian cells [1,11–13]. PHMB has been loaded into different types of wound dressings, e.g., gauze and bandages [14,15], foam [16,17], nanofibrous membranes of cellulose acetate and polyester urethane

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0928-4931/

[18], or BC dressings [10,19,20]. Some of BC dressings loaded with PHMB are commercially available.

To improve the effectiveness of this BC dressing, we here introduce another bioactive factor for an enhancement of the re-epithelialization process which is one of the critical steps in wound healing process. Silk sericin (SS) is chosen for this purpose. SS (molecular weight of 20–310 kDa) is an adhesive protein that binds two fibroin strands in silk fibers [21]. It is a hydrophilic, biocompatible, biodegradable, negatively-charged material [22,23] with various biological properties [24]. It has been used to promote proliferation of human fibroblasts [25] or human epithelial HeLa cells [26] and prevent the UVB-induced apoptosis in human skin keratinocytes [27]. The addition of SS to culture media enhances collagen type I production by the L929 mouse fibroblast cell line [23] and promotes L929 migration [28]. An animal model revealed that SS-treated wounds had reduced inflammatory reactions and faster healing time than Betadine®-treated wounds [29]. In the clinical tests of split-thickness skin wounds, the complete healing time of wounds treated with a SS-releasing dressing was significantly shorter than that required for wounds treated with Bactigras® [30]. Furthermore, SS was shown to enhance re-epithelialization [23,28–33].

To our design, the combination of SS and PHMB into a BC wound dressing should capture the unique benefits of both SS and PHMB. In this study, the interactions between these components that affect the biological properties of BC dressing were investigated. The interaction between PHMB and SS was evaluated by attenuated total reflection Fourier Transform Infrared spectroscopy (ATR-FTIR). The loading quantities and sequence of both compounds were optimized. The antimicrobial test was performed by both the disc diffusion and broth dilution techniques using both Gram-positive (*Bacillus subtilis*, *Staphylococcus aureus*, and methicillin-resistant *S. aureus*) and Gram-negative bacteria (*Escherichia coli*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*) to determine minimum bactericidal concentration of PHMB. Then, the SS loading amount was varied to achieve an effective released amount which was suitable for collagen-I production by fibroblast cells. The in vitro release of PHMB and SS from the BC dressing was also evaluated. Finally, the effects of the dual-loaded SS/PHMB released from the BC dressing on the antimicrobial effect were compared with other commercially available antimicrobial wound dressings.

2. Materials and methods

2.1. Materials

Coconut water was obtained from coconuts purchased locally. Ammonium phosphate ($(NH_4)_3PO_4$), glacial acetic acid (CH_3COOH), and other chemicals used were of analytical grade (Sigma-Aldrich, USA). Polyhexamethylene biguanide was kindly provided by Lonza Group Ltd. (Basel, Switzerland). Silk sericin was extracted according to a high temperature and high pressure degumming method described in a previous report [23]. Proteoglycan-IPC, a soluble PG extracted from the nasal cartilage of *Oncorhynchus keta* (Salmonidae), was purchased from Icimaru Pharcos Co., Ltd. (Gifu, Japan).

2.2. Preparation of biocellulose

Biocellulose was prepared from a static culture of an *Acetobacter xylinum* strain (Kasetsart University, Bangkok, Thailand) which was isolated from nata de coco, using a coconut water-based medium. The preparation of the medium was slightly modified from the method developed by Verschuren et al. [34]. One liter of coconut water was boiled and supplemented with 50 g of sucrose, 5 g of $(NH_4)_3PO_4$, and 10 mL of CH_3COOH. The pH of the medium was adjusted to 4.5 with CH_3COOH. Then, 10 mL of *A. xylinum* was added into the pH-adjusted coconut water based medium and poured into molds for fermentation. The fermentation was carried out under sterilized static conditions at 30 °C for 3–5 days to pre-culture the *A. xylinum* strain. After obtaining the stock solution of *A. xylinum*, the fermentation was incubated at 30 °C under static conditions for another 7 days to form the BC. The formed bacterial nanocellulose gels were washed with 2% aqueous NaOH solution at 70 °C for 10 min, and then washed repeatedly until a neutral pH was obtained.

2.3. Preparation of the BC wound dressing containing bioactive compounds

The BC wound dressing was loaded with PHMB and SS. Proteoglycan (PG) which is known as a healing-promoting agent was used as a control for SS. All samples prepared in this study were summarized in Table 1. Each dressing was loaded with either one or two components. In the first study, the BC wound dressing was immersed into PHMB solutions with different concentrations (0.0125–0.6% w/v) for 2 h to determine the minimum bactericidal concentration (MBC) of PHMB loaded into the BC wound dressing.

The second experiment was intended to study the interaction between PHMB and two compounds (SS or PG), the loading sequence of each compound and MBC of PHMB in the dual-loaded BC. The BC wound dressing was immersed in a solution of the first compound for 2 h. Subsequently, the soaked BC was allowed to dry before being soaked in a solution of the second compound for another 2 h. The dual-loaded BC was air-dried before the tests. The PHMB concentration was varied from 0.2–0.4% w/v while the concentration of SS or PG was fixed at 1% w/v.

After the MBC of PHMB was obtained, the loading amount of PHMB was then optimized. In this experiment, SS was first loaded into the BC (the size of BC was $10 \times 10 \times 0.01$ cm^3) before PHMB. The loading amount of SS was fixed at 2 mL of SS solution (1% w/v) per side of the BC wound dressing (or total loading of 4 mL). After 2 h of SS adsorption, PHMB was added into the BC/SS wound dressing; the loading amount ranged from 3 to 6 mL of PHMB solution (0.3% w/v) per side of BC/SS (or total loading was from 6 to 12 mL). Before the tests, the dual-loaded BC was air-dried for 2 h.

Finally, the loading amount of SS on BC was optimized for the effective release amount of sericin. In this experiment, SS was first loaded into BC with different loading amounts, ranging from 2 to 3 mL of SS solution (1% w/v) per side of the BC wound dressing (or total loading of 4 to 6 mL). After 2 h of SS adsorption, the PHMB solution (0.3% w/v) was added to the BC/SS wound dressing at a fixed amount of 5 mL per side of BC/SS or total loading of 10 mL. The dual-loaded BC was air-dried for 2 h before the in vitro release test of SS. In this experiment, the single loaded PHMB on BC was used as a negative control for the SS release experiment.

2.4. Antimicrobial efficacy test

Antimicrobial efficacy of different types of PHMB loaded on BC was evaluated in triplicate by the disc diffusion method (CLSI M2-A9) and the broth dilution method (CLSI M7-A7). Six strains of bacteria were selected for these tests: *B. subtilis* (ATCC 6633, Gram-positive), *S. aureus* (ATCC 25923, Gram-positive), methicillin-resistant *S. aureus* (MRSA, Gram-positive), *E. coli* (ATCC 25922, Gram-negative), *A. baumannii* (ATCC 19606, Gram-negative), and *P. aeruginosa* (ATCC 27853, Gram-negative). Müller Hinton (MH) agar was used for culture, inoculation and antimicrobial efficacy tests. All bacterial strains were cultured on an agar plate at 37 °C for 24 h right before preparation of the inoculum. The inoculum was prepared by selecting three to five isolated colonies of bacteria into 5 mL of Tryptone Soya Broth (TSB), and followed by incubation at 37 °C for 4–6 h. The content of bacteria was verified by a UV/VIS spectrometer (Lambda 25, Perkin Elmer, Waltham, MA, USA) at 625 nm. The absorbance of the inoculum should be between 0.08 and 0.13 for a bacterial content of 1.5×10^8 CFU/mL.

For the disc diffusion method (CLSI M2-A9), one swab was applied on the entire surface of the MH agar plate. Then, the BC containing PHMB ($1 \times 1 \times 0.1$ cm^3) was placed on the MH agar plate and incubated

Table 1
Summary of components loaded in different BC wound dressings used in this study.

Experimental aim(s)	Sample	1st bioactive compound loading		2nd bioactive compound loading	
		Compound	Conc./amount	Compound	Conc./amount
Effective PHMB concentration in BC	PHMB	PHMB	0.0125–0.6%	N/A	–
Interaction study between two compounds & effective PHMB concentration in the dual loaded BC	SS	Silk sericin	1%	N/A	–
	PG	Proteoglycan	1%	N/A	–
	SS/PHMB	Silk sericin	1%	PHMB	0.2–0.4%
	PHMB/SS	PHMB	0.2–0.4%	Silk sericin	1%
	PG/PHMB	Proteoglycan	1%	PHMB	0.2–0.4%
	PHMB/PG	PHMB	0.2–0.4%	Proteoglycan	1%
Optimization of PHMB loading amount in the dual loaded BC	SS2/PHMB3	Silk sericin	2 mL	PHMB[a]	3 mL
	SS2/PHMB4	Silk sericin	2 mL	PHMB[a]	4 mL
	SS2/PHMB5	Silk sericin	2 mL	PHMB[a]	5 mL
	SS2/PHMB6	Silk sericin	2 mL	PHMB[a]	6 mL
Optimization of SS loading amount in the dual loaded BC	PHMB5	PHMB[a]	5 mL	N/A	–
	SS2/PHMB5	Silk sericin	2 mL	PHMB[a]	5 mL
	SS3/PHMB5	Silk sericin	3 mL	PHMB[a]	5 mL

[a] Concentration of PHMB for loading on BC was 0.3% w/v

at 37 °C for 24 h. The distance between each BC was much higher than the inhibition zone. After incubation, the size of the inhibition zone was immediately measured. The size of the inhibition zone was reported as the diameter with reference to the diagonal length of the BC square.

For the broth dilution method (CLSI M7-A7), the inoculum was diluted to achieve a bacterial concentration of 5×10^5 CFU/mL before placing the BC containing different amounts of PHMB in a test tube. After 24 h of incubation at 37 °C, one swab of the broth was applied to the entire surface of the MH agar plate. The agar plate was then incubated for another 24 h at 37 °C before evaluating the MBC, which was based on no observation of bacterial colony on the agar plate.

2.5. Attenuated total reflection Fourier Transform Infrared spectroscopy

Attenuated total reflection Fourier Transform Infrared (ATR-FTIR) spectroscopy was employed to investigate the molecular interactions between PHMB and SS or PG molecules. The spectra of pure compounds and their mixtures were collected by a Nicolet 6700 FTIR spectrometer coupled to a continuum FTIR microscope and a mercury-cadmium-telluride (MCT) detector. A homemade slide-on Ge micro internal reflection element (μIRE) accessory was employed for all spectral acquisitions. The IRE was aligned and fixed at the optimum position of the slide-on housing where the contact tip coincided with the focal point of the built-in 15× Cassegrain infrared objective lens. The spectral acquisitions were performed in the reflection mode of infrared spectroscopy. A sample was prepared by dropping 1% of SS, 1% of PG, 0.3% of PHMB, and their mixture sample solutions on a glass slide and drying them using a vacuum pump at room temperature. For spectral acquisition of the sample, a dried sample on a glass slide was brought into contact with the IRE. All spectra were collected with 256 scans at a resolution of 4 cm^{-1}. Each sample was examined at several points for comparison purposes.

2.6. In vitro release test of PHMB and SS

The BC wound dressing was cut to a size of $1 \times 1 \times 0.1$ cm^3 and placed in triplicate in a vessel containing 3 mL of phosphate-buffered saline solution (PBS, pH 7.4) at 37 °C. The PBS solutions were collected at pre-determined time points (0.5, 2, 4, 8, 24, 48, 72 and 168 h) and shaken before measurement. In the SS case, the BCA protein assay kit (Pierce, Rockford, IL, USA) was used to measure the released amount of SS protein. The absorbance of the solution was measured with an UV/VIS spectrometer at a wavelength of 562 nm. The amount of SS was determined from a standard curve prepared from different concentrations of bovine serum albumin. For PHMB, the solution absorbance was measured with the same spectrometer at a wavelength of 235.5 nm. The concentrations of released PHMB at different times were calculated by evaluating the solution absorbance against a standard curve, prepared from different known PHMB concentrations.

2.7. Preparation of fibroblast cell for collagen type 1 production test

The fibroblast cells were produced with slightly modified method of our previous work [23]. Briefly, Dulbecco Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and antibiotics (100 U penicillin and 100 U streptomycin per mL) was used to culture the mouse fibroblast cell lines L929 which were purchased from the Chinese Academy of Preventive Medical Sciences, Beijing, China under 5% CO_2 at 37 °C. The medium was replaced every 2 days. Then, cells were collected by using 0.25% trypsin-EDTA (Gibco®, California, USA) and added fresh culture medium to create a new single cell suspension for further incubation.

2.8. Collagen type 1 production test

The 2.5×10^4 cells/well of mouse fibroblast cell lines L929 were seeded in a 48-well plate DMEM solution containing 10% FBS. After 24 h, the mediums were replaced with released solution of SS2/PHMB5 and BC wound dressing which were filter sterilized by 0.22 μm membrane filters (triplicate samples). After 30 min, the mediums were changed with DMEM containing the same sericin concentration as the 24 h released sericin solution from SS2/PHMB5 dressing. After 3 days, the total amounts of soluble collagen type 1 were assayed by using the Sircol® collagen assay kit (Biocolor Ltd., Northern Ireland, UK). Microplate reader (Biohit 830, Biohit®, Helsinki, Finland) was used for detection of the outcomes at 500 nm. The results were calculated compared with standard curve of soluble collagen which was prepared from standard bovine collagen type 1.

2.9. Statistical analysis

All statistical evaluations were performed using SPSS version 17.0 (SPSS. Co., Ltd., Bangkok, Thailand). Differences in the data were considered statistically significant at $p < 0.05$, using one way ANOVA.

3. Results and discussion

3.1. PHMB minimum bactericidal concentration used for loading into the BC wound dressing

The BC wound dressing was immersed in different concentrations of PHMB (0.0125%, 0.025%, 0.05%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.6% w/v) for

2 h to determine the effective concentration for antimicrobial activity. The broth dilution technique was employed for this evaluation.

Table 2 shows the PHMB concentration which killed all bacteria entirely as no colonies were observed after 24 h when one swab of the bacterial broth (which had been incubated with BC loaded with different PHMB concentrations for 24 h at 37 °C) was applied on the MH agar plate and culture continued for 24 h at 37 °C. The MBC of PHMB was 0.2% w/v for effective bactericidal activity. The highest PHMB concentration was needed to kill MRSA in the group of the Gram-positive bacteria and *P. aeruginosa* for the Gram-negative case. This concentration range was close to the reported values of commercial wound dressings: 0.2% PHMB in Kerlix AMD™ antimicrobial gauze and bandages [14], 0.3% PHMB in Suprasorb® X + PHMB, and 0.5% PHMB in the Kendall™ AMD antimicrobial foam [16].

3.2. Interaction study between two compounds and the effective PHMB concentration in the dual loaded BC wound dressing

The BC wound dressing was loaded with two different compounds to evaluate the dual release of two bioactive compounds: PHMB for its antimicrobial activity, and either SS or PG to enhance wound repair. However, the interaction between the two bioactive compounds needed to be investigated in terms of the loading sequence and loading amount since they could impair the antimicrobial activity, which is the main requirement of using a BC dressing to treat infected wounds. In this test, only *P. aeruginosa* was selected because it had the highest resistance to PHMB, as shown in the previous section. The single loaded PHMB dressing was used as a positive control, while the single loaded SS and PG dressings were used as the negative control.

Fig. 1 shows the results from the disc diffusion test regarding the *P. aeruginosa* inhibition zone distance, influenced by different types of BC wound dressings (note; SS/PHMB: silk sericin was loaded into the dressing before PHMB; PHMB/SS: PHMB was loaded into the dressing before SS; PG/PHMB: proteoglycan was loaded into the dressing before PHMB; PHMB/PG: PHMB was loaded into the dressing before PG; PHMB: this dressing was only loaded with PHMB; SS: this dressing was only loaded with SS; and PG: this dressing was only loaded with PG). The effects of the loading sequences and the interactions among all ingredients were obvious. There was no antimicrobial activity which could be seen in the SS, PG, PHMB/SS, PG/PHMB, and PHMB/PG dressings. For the dual-loaded dressing, the antimicrobial activity was only observed in the SS/PHMB dressing. The larger SS molecules (MW = 20–310 kDa, [29]) could potentially block the smaller PHMB molecules (MW = 3 kDa, [1]) if PHMB was loaded before SS in the case of the PHMB/SS dressing (see Fig. 2B for a graphical illustration). This situation might hinder PHMB from reacting with the bacterial membrane, compared with the SS/PHMB dressing. In the SS/PHMB dressing, there would be higher amount of PHMB available for inhibiting bacterial activities (Fig. 2A). There was some interaction between SS and PHMB because the effective concentration of PHMB was increased to 0.3% w/v, compared to 0.2% w/v in the single PHMB loaded dressing. However, this interaction between SS and PHMB was probably weak because PHMB still maintained its antimicrobial activity with a higher loaded PHMB concentration.

Table 2
The PHMB minimal bactericidal concentrations of the tested bacteria, observed after applying one swab of bacterial broth dilution (which had incubated with BC loaded with different PHMB concentrations for 24 h at 37 °C) on the MH agar plate and continued culture for 24 h at 37 °C.

Bacteria strain	PHMB minimal bactericidal concentration for loading into the BC wound dressing (% w/v)
B. subtilis	0.025
S. aureus	0.050
MRSA	0.100
E. coli	0.050
A. baumannii	0.025
P. aeruginosa	0.200

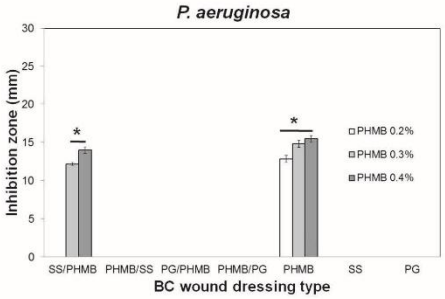

Fig. 1. The inhibition zone distance against *P. aeruginosa*, 24 h after placing different types of loaded BC wound dressings on a MH agar plate, which was cultured at 37 °C (* indicates statistical difference between the pair, $p < 0.05$).

In the case of the proteoglycan-containing dressings (PG/PHMB and PHMB/PG), PHMB was ineffective at inhibiting *P. aeruginosa*, regardless of the loading sequence. PHMB might have been caught in the highly branched PG network (Fig. 2C & D). In addition, the positively charged PHMB might form strong interactions with the highly negatively-charged PG molecules [12,13]. Müller et al. [35] also found that highly negatively-charged glycosaminoglycan (chondroitin sulfate) completely neutralized the antimicrobial effects of PHMB against *S. aureus*, *Enterococcus faecium*, and *E. coli*.

The electrostatic force was found to be a major interaction between positively-charged PHMB and other charged molecules. The attraction force was reported in the case of PHMB with a negatively-charged plant cellulose [36] through hydrogen bonding. Dilamian et al. [37] reported that PHMB formed an attractive interacteraction with negatively-charged poly(ethylene oxide) and a repulsive interaction with and positively-charged chitosan; however these electrostatic interactions did not impair the antimicrobial activity of PHMB. The electrostatic interaction issue would be elevated if PHMB interacts with highly negatively-charged molecules such as PG (reported in this work) or chondroitin sulfate (reported by Müller et al. [35]). The antimicrobial activity of PHMB might be totally ineffective for certain bacterial strains which are difficult to treat.

3.3. ATR-FTIR study investigating the interaction between two compounds

In order to understand the interaction details, ATR-FTIR was employed to evaluate the molecular interaction between two compounds (PHMB vs. SS, and PHMB vs. PG).

In Fig. 3A, the pure SS sample shows three peaks at 1650, 1624, and 1518 cm^{-1} attributed to amide I, amide I, and amide II, respectively, which are characteristic of protein. When the SS sample was mixed with the PHMB sample, the peak shoulder at 1650 cm^{-1} (in the spectrum of the mixture) of amide I (from SS molecules) slightly decreased, which indicates a change in the secondary structure of the protein [2]. The amide I peak at 1624 cm^{-1} was unaffected. However, the band at 1544 cm^{-1} (in the spectrum of the mixture) was a combination of the peaks at 1545 and 1518 cm^{-1} attributed to the C=N stretching vibration mode of PHMB molecules and amide II of SS molecules, respectively. It seems that there was no interaction between C=N of PHMB molecules and amide II of SS molecules. These results also suggest that there was a weak interaction between SS and PHMB molecules which slightly affected the amide I region or the secondary structure of the protein. This finding corresponds to the previous conclusion of the bacterial test that SS only had a weak interaction with PHMB, necessitating

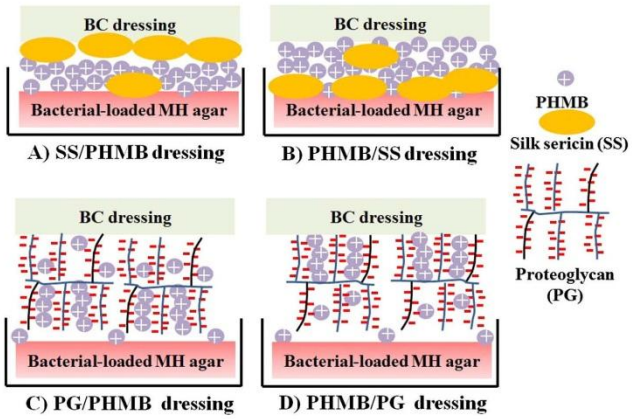

Fig. 2. An illustration of the loading sequence of bioactive compounds: positively charged PHMB, SS, and highly negatively-charged PG into the neutral BC wound dressing: A) SS/PHMB dressing in which SS was loaded into the dressing before PHMB, B) PHMB/SS dressing in which PHMB was loaded into the dressing before SS, C) PG/PHMB dressing in which PG was loaded into the dressing before PHMB, and D) PHMB/PG dressing in which PHMB was loaded into the dressing before PG.

a higher PHMB concentration (0.3% w/v compared to 0.2% w/v in single loaded PHMB dressing).

In Fig. 3B, the pure PG sample shows a peak at 1650 cm^{-1} attributed to C═O stretching vibration mode and the pure PHMB sample shows a peak at 1545 cm^{-1} attributed to C═N stretching vibration mode. In the mixture of PG and PHMB, the C═O peak slightly shifted from 1650 cm^{-1}, compared to the pure PG sample, while the C═N peak significantly shifted to 1558 cm^{-1} from 1545 cm^{-1} in the pure PHMB sample. This shift indicates that there was a strong molecular interaction between the C═O functional group of PG molecules and the C═N functional group of PHMB molecules in the mixture [38]. It is possible that lone pair electrons from the C═O of PG formed a hydrogen bond with H–N═C of PHMB, as shown in Fig. 4.

This finding is in agreement with those reported by Zhang and Magnes, who indicated that peak shifts are caused by hydrogen bonding [39,40]. Moreover, the precipitation of PG and PHMB could be observed when the PG solution was mixed with the PHMB solution during the preparation of the PG/PHMB mixture. The precipitation might result from electrostatic interactions between the negatively charged functional group of PG and positively charged PHMB. From the above findings, it can be concluded that there was a strong molecular interaction between PHMB and PG molecules. This strong interaction could explain the previous results in Section 3.2 in that there was no antimicrobial activity found in the PG/PHMB and PG/PHMB dressings (Fig. 1).

In addition, the interaction between BC and other molecules (SS, PHMB, and PG) could not be observed as a change in the ATR-FTIR spectra (data was not shown here). The interaction was suspected to be a hydrogen bond between the –OH groups of BC and the C═O groups of the protein in SS, H–N═C in PHMB, or C═O/–OH groups in PG, as shown in Fig. 5. However, the strength of these interactions might not be strong enough to shift the peak of BC due to the molecular size and steric hindrance of structures.

In summary, PG should not be loaded with PHMB if antimicrobial activity is required against various bacterial strains. The dual loading system of SS and PHMB into the BC dressing was still effective if SS was loaded before PHMB. In additon, the concentration of PHMB must be at least 0.3% w/v to maintain antimicrobial effectiveness.

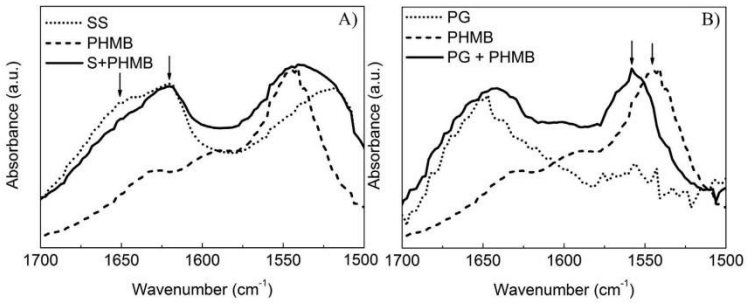

Fig. 3. ATR-FTIR spectra: A) shows the spectra of pure SS, pure PHMB, and the SS and PHMB mixture (SS + PHMB) while B) shows the spectra of pure PG, pure PHMB, and the PG and PHMB mixture (PG + PHMB), the downward arrows indicating a noticeable difference between the mixture and either pure SS or pure PHMB.

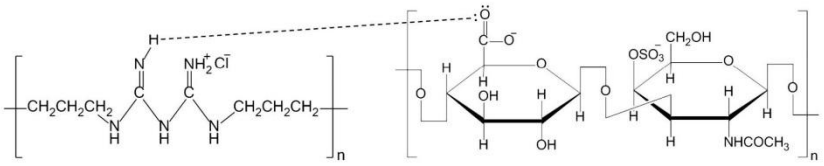

Fig. 4. An illustration of hydrogen bonding between PHMB and chondroitin sulfate A, which is a part of the PG molecule.

3.4. Optimization of the PHMB loading amount in pre-adsorbed SS on the BC wound dressing

P. aeruginosa was the only bacterial strain selected for the optimization of the PHMB (0.3% w/v) loading amount on pre-adsorbed SS (2 mL/side of a 1% w/v SS solution) on the BC wound dressing, since this strain showed the highest resistance to the antimicrobial activity of PHMB. Fig. 6 illustrates the results from the disc diffusion test of the inhibition zone size, as influenced by different PHMB loading amounts on pre-adsorbed SS on the BC wound dressing against *P. aeruginosa*. PHMB solution (0.3% w/v) needed to be loaded in a minimum amount of 4 mL per side of the wound dressing for effective inhibition of *P. aeruginosa* growth. The dressing loaded with 5 mL and 6 mL of PHMB solution showed significantly higher inhibition zone than that loaded with 4 mL of PHMB solution. The broth dilution results also indicated that the loading of 4 mL of PHMB solution (0.3% w/v) was essential for complete elimination of *P. aeruginosa*.

3.5. Optimization of the SS loading amount in the dual loaded BC for effective SS release in PBS

This experiment was intended to determine the right SS loading amounts into the BC wound dressing, so the optimal concentration of the released SS was at 100 μg/mL to promote fibroblast collagen production, without losing cell viability [23]. Fig. 7 illustrates the released profile of SS in PBS. For both formulations (SS2/PHMB5 and SS3/PHMB5), an initial burst release of SS was observed. In the first 4 h, the concentration of SS reached the effective level (100 μg/mL) to promote fibroblast collagen production. A steady release period was observed from 24 h onward. During the whole test, there were no statistical differences between the SS2/PHMB5 and SS3/PHMB5 formulations at the same time points. The SS2/PHMB5 formulation was preferable over SS3/PHMB5 in terms of promoting fibroblast collagen production, since the released amount was closer to100 μg/mL.

3.6. Collagen type 1 production from SS2/PHMB5 and BC wound dressing

The enhancement of collagen type 1 produced by fibroblast cells cultured with the released solution of SS2/PHMB5 formulation was elucidated in Fig. 8. It was shown that the cells cultured with the released solution of SS2/PHMB5 formulation produced significantly higher amount of collagen type 1 than those cultured with the BC wound dressing. This data supported our results that SS which released from SS2/PHMB5 steadily at around 100 μg/mL could efficiently induce the production of collagen type 1 by fibroblast cells.

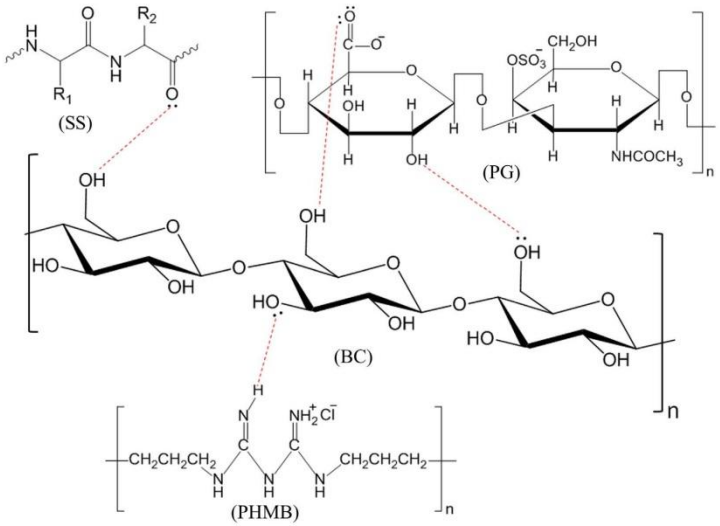

Fig. 5. An illustration of hydrogen bonding between BC and other molecules (SS protein, PHMB, and PG).

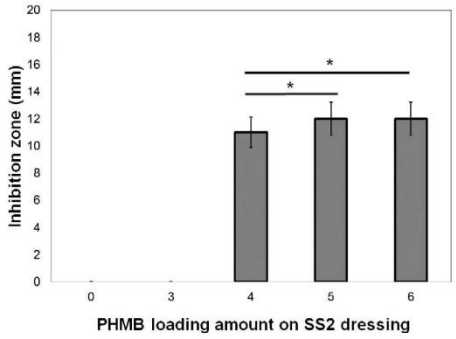

Fig. 6. The effects of different PHMB loading amounts (mL per side) loaded on the BC wound dressing which was pre-adsorbed with 2 mL/side of SS solution (1% w/v) on the inhibition zone distance against *P. aeruginosa*, 24 h after placing the BC dressing containing PHMB on a bacteria-loaded MH agar plate, cultured at 37 °C (* indicates a statistical difference ($p < 0.05$) between the pair).

3.7. Minimum bactericidal concentration of the PHMB solution

Before the in vitro release of PHMB from the BC wound dressings, the minimum PHMB concentration was evaluated for its antimicrobial activity against various bacterial strains by the broth dilution technique. In this test, the MBC was evaluated directly with PHMB solutions at different concentrations. There were no BC wound dressings involved in these tests, unlike in Section 3.1. These test results were used as a guideline for selecting the effective formulation of the BC dressing. The concentration of the released PHMB must be at least equal to the minimum bactericidal concentration, obtained in these tests. Table 3 summarizes the PHMB concentration which killed all bacteria entirely as no colonies were observed after 24 h when one swab of bacterial broth dilution (which had been incubated with different PHMB concentrations for 24 h at 37 °C) was applied on the MH agar plate and culture was continued for 24 h at 37 °C.

The concentration of PHMB must be at least 0.005% w/v to be able to kill all bacterial strains. Previously Müller et al., reported that a PHMB concentration of 0.005% is effective for the extermination of *S. aureus* and *E. coli* [41].

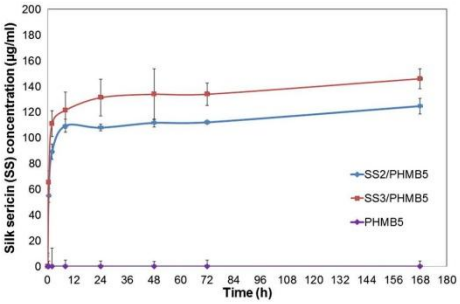

Fig. 7. The amount of SS released in a PBS solution from different types of BC wound dressings: SS2/PHMB5 [2 mL/side of SS solution (1% w/v) loaded onto the BC wound dressing before adding 5 mL/side of PHMB solution (0.3% w/v)], SS3/PHMB5 [3 mL/side of SS solution (1% w/v) loaded onto the BC wound dressing before adding 5 mL/side of PHMB solution (0.3% w/v)], and PHMB5 [5 mL/side of PHMB solution (0.3% w/v) only loaded in the BC wound dressing] (there were no statistically significant differences in the amount of SS released from SS2/PHMB5 and SS3/PHMB5 formulations at the same time points).

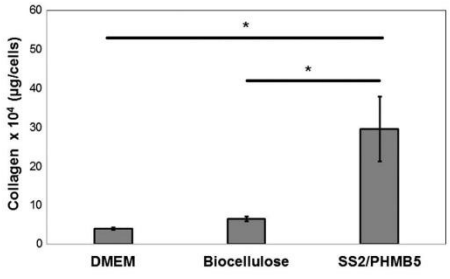

Fig. 8. The amount of collagen type 1 in released solution of SS2/PHMB5 and BC wound dressing; SS2/PHMB5 [2 mL/side of SS solution (1% w/v) loaded onto the BC wound dressing before adding 5 mL/side of PHMB solution (0.3% w/v)] (* indicates a statistical difference ($p < 0.05$) between the pair).

3.8. In vitro release of PHMB in PBS

Fig. 9 shows the amount of PHMB released from different types of BC wound dressings during the week-long testing period. After only 30 min, the concentrations of released PHMB in PBS were higher than 0.005% w/v, as observed from all BC wound dressings (SS2/PHMB5, SS3/PHMB5, and PHMB5). This concentration was sufficient for effective elimination of all bacterial strains as found in the previous test (Section 3.7). The PHMB concentration in PBS released from the SS2/PHMB5 dressing was found to be insignificantly higher than the amount released from the SS3/PHMB5 dressing at all time points during the test; this was because there was a weak interaction between SS and PHMB molecules, which slightly affected the secondary structure of the protein in the amide I region (Fig. 3A). The PHMB concentration released from the PHMB5 dressing was significantly higher than the level released from the SS2/PHMB5 and SS3/PHMB5 dressings ($p < 0.05$).

From in vitro release results of both SS and PHMB, it could be concluded that the formulation of SS2/PHMB5 was better than SS3/PHMB5 since the released SS concentration was close to the optimal value which enhances fibroblast collagen production. In addition, the release of PHMB was slightly higher in the SS2/PHMB5 formulation than the SS3/PHMB5 formulation.

3.9. The comparison of antimicrobial activities between the dual loaded SS/PHMB BC wound dressing (SS2/PHMB5) and the single loaded PHMB BC wound dressing (PHMB5)

The dual-loaded SS/PHMB BC wound dressing (SS2/PHMB5) had more benefit than the single loaded PHMB BC wound dressing (PHMB5), since SS released from the SS2/PHMB5 wound dressing enhanced fibroblast collagen production. In Section 3.2, the interaction

Table 3
Minimal bactericidal concentrations of PHMB solution against the tested bacteria, observed after applying one swab of bacterial broth dilution (which had incubated with different PHMB concentration for 24 h at 37 °C) on the MH agar plate and continued culture for 24 h at 37 °C.

Bacteria strain	Minimal bactericidal concentration of PHMB solution (% w/v)
B. subtilis	0.00375
S. aureus	0.00150
MRSA	0.00150
E. coli	0.00150
A. baumannii	0.00375
P. aeruginosa	0.00500

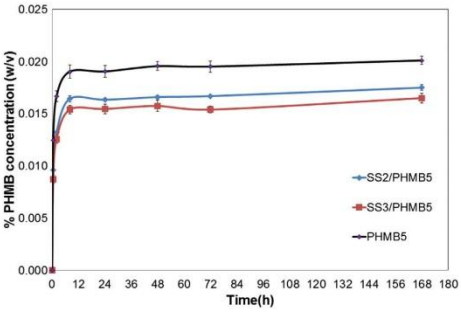

Fig. 9. The amount PHMB released into PBS solution from different types of BC wound dressings: SS2/PHMB5 [2 mL/side of SS solution (1% w/v) loaded onto the BC wound dressing before adding 5 mL/side of PHMB solution (0.3% w/v)], SS3/PHMB5 [3 mL/side of SS solution (1% w/v) loaded onto the BC wound dressing before adding 5 mL/side of PHMB solution (0.3% w/v)], and PHMB5 [5 mL/side of PHMB solution (0.3% w/v) only loaded onto the BC wound dressing] (there were no statistically significant differences in the amount of PHMB released from SS2/PHMB5, SS3/PHMB5 and PHMB5 formulations at the same time points).

between SS and PHMB was found to have minimal negative effects on PHMB antimicrobial activity against *P. aeruginosa* if SS was loaded before PHMB in the BC wound dressing. However, there might be some variation against other bacterial strains. In this experiment, the antimicrobial effectiveness of dual-loaded SS2/PHMB5 was compared to the PHMB5 formulation against all bacterial strains using both discs the diffusion and broth dilution techniques.

Fig. 10 shows the comparison of antimicrobial activity between the dual loaded SS/PHMB BC wound dressing (SS2/PHMB5) and the single loaded PHMB BC wound dressing (PHMB5) against all bacterial strains. There were no statistical differences in the inhibition zone observed between the two types of wound dressing with the same bacteria. This finding confirmed that the PHMB antimicrobial activities against all tested bacteria were not compromised by the addition of SS, if it was loaded first in the BC wound dressing before PHMB. Based on the broth dilution results, there were no differences in the PHMB antimicrobial activity observed between the SS2/PHMB5 and PHMB5 formulation wound dressings even after the agar plates were left for 72 h.

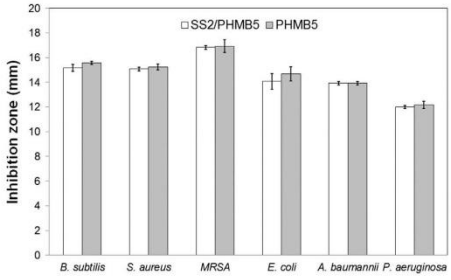

Fig. 10. The comparison of antimicrobial activity between two wound dressings: the dual loaded SS/PHMB BC wound dressing (SS2/PHMB5) and the single loaded PHMB BC wound dressing (PHMB5) against all tested bacteria (*B. subtilis*, *S. aureus*, MRSA, *E. coli*, *A. baumannii*, and *P. aeruginosa*), 24 h after placing the BC dressings containing PHMB on bacteria-loaded MH agar plates which were cultured at 37 °C (there were no statistically significant differences in the inhibition zone observed between the two types of wound dressing using the same bacteria).

3.10. The comparison of the antimicrobial activity of the BC wound dressing (SS2/PHMB5) with other commercially available antimicrobial wound dressings

To evaluate the potential of commercialization of our novel BC wound dressing, the comparison of antimicrobial activity was conducted between the SS2/PHMB5 wound dressing and other commercially available antimicrobial dressings such as Bactigras® (Smith & Nephew plc., London, UK), Acticoat® (Smith & Nephew plc), and Suprasorb®X + PHMB.

It is clear from Fig. 11 that SS2/PHMB5 is far superior in terms of antimicrobial activity against all bacterial strains than Bactigras, a paraffin-impregnated gauze dressing containing chlorhexidine acetate. In comparison with silver-loaded Acticoat®, the antimicrobial activity of the SS2/PHMB5 dressing was better against Gram-positive bacteria often found in chronic wounds (*S. aureus* and MRSA). In the case of Gram-negative bacteria, the antimicrobial activity of the SS2/PHMB5 dressing was comparable to that of Acticoat®, but had less of an effect on inhibiting *P. aeruginosa* growth. The antimicrobial difference between the SS2/PHMB5 dressing and Suprasorb®X + PHMB was only noticed for *B. subtilis*. However, it is inconclusive that Suprasorb®X + PHMB would be better than the SS2/PHMB5 dressing for treating chronically infected wounds, because *B. subtilis* has not been reported in infected wounds [42,43]. These results suggest that the antimicrobial activity of the SS2/PHMB5 dressing is comparable to that of commercially available dressings. There was no single dressing which was more effective than others for treating all tested bacteria. The advantage of SS2/PHMB5 over the commercial dressings is the promotion of wound healing from SS.

Comparing to the report on BC dressing of other studies, our SS2/PHMB5 dressing would show higher potential in term of the enhancement of collagen type 1 production than the single-loading of antiseptics in BC dressings, such as silver/silver chloride nanoparticles [44–52], silver sulfadiazine [5], benzalkonium chloride [53], chitosan [54,55], and PHMB [56]. Considering the study that loaded two compounds into BC dressing (negatively-charged gold nanoparticles and positively-charged lysozyme [57], or negatively-charged phosvitin and positively-charged chitosan [58] loaded into negatively-charged cellulose acetate by a layer-by-layer self-assembly technique), the goal of the compounds loaded was difference from ours. Best on our best knowledge, this study is the first report of the release of the antiseptic and the bioactive compound simultaneously from BC wound dressing for the antimicrobial activity and acceleration of wound healing.

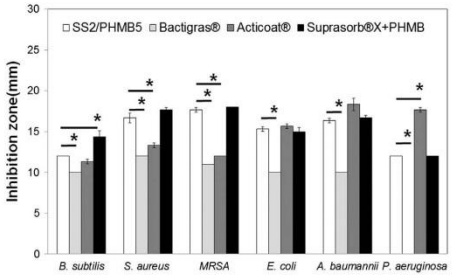

Fig. 11. A comparison of the antimicrobial activity of the BC wound dressing developed in this work (SS2/PHMB5) with commercially available antimicrobial wound dressings [Bactigras®, Acticoat® and Suprasorb®X + PHMB] against all tested bacteria (*B. subtilis*, *S. aureus*, MRSA, *E. coli*, *A. baumannii*, and *P. aeruginosa*), 24 h after placing the BC dressings containing PHMB on bacteria-loaded MH agar plates which were cultured at 37 °C (* indicates a statistically significant difference ($p < 0.05$) between the SS2/PHMB5 dressing and other commercial dressings).

4. Conclusions

We have successfully developed a BC wound dressing loaded with SS, a compound known to enhance fibroblast collagen production without negative impacts on the antimicrobial effects of PHMB. The loading concentration of PHMB needed to be at least 0.3% w/v for effective elimination of all tested bacteria (*B. subtilis*, *S. aureus*, MRSA, *E. coli*, *A. baumannii*, and *P. aeruginosa*). For dual loading, the type of compound and the loading sequence could have an undesirable negative impact on PHMB antimicrobial activity. Highly negatively-charged PG completely impaired PHMB antimicrobial activity against certain bacteria that are difficult to treat, e.g., *P. aeruginosa*. The loading sequence was crucial in the formulation containing PHMB and SS. Silk sericin needed to be loaded first to maintain the antimicrobial effects of PHMB. The SS2/PHMB5 formulation was found to be the best, since it released SS at a concentration close to the optimal conditions to enhance fibroblast collagen production and released PHMB at a higher concentration than the other dual formulations. In addition, the antimicrobial activity of SS2/PHMB5 was comparable to that of commercially available antimicrobial wound dressings.

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ORIGINAL PAPER

The safety and efficacy of bacterial nanocellulose wound dressing incorporating sericin and polyhexamethylene biguanide: in vitro, in vivo and clinical studies

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Abstract In our previous work, we have attempted to develop a novel bacterial nanocellulose wound dressing which composed of both polyhexamethylene biguanide (PHMB) as an antimicrobial agent and sericin as an accelerative wound healing component. The loading sequence and concentration of PHMB and sericin were optimized to provide the wound dressing with the most effective antimicrobial activity and enhanced collagen production. In this study, further in vitro, in vivo, and clinical studies of this novel wound dressing were performed to evaluate its safety, efficacy, and applicability. For the in vitro cytotoxic test with L929 mouse fibroblast cells, our novel dressing was not toxic to the cells and also promoted cell migration as good as the commercially available dressing, possibly due to the component of sericin released. When implanted subcutaneously in rats, the lower inflammation response was observed for the novel dressing implanted, comparing to the commercially available dressing. This might be that the antimicrobial PHMB component of the novel dressing played a role to reduce infection and inflammation reaction. The clinical trial patch test was performed on the normal skin of healthy volunteers to evaluate the irritation effect of the dressing. Our novel dressing did not irritate the skin of any volunteers, as characterized by the normal levels of erythema and melanin and the absence of edema, papule, vesicle, and bullae. Then, the novel dressing was applied for the treatment of full-thickness wounds in rats. The wounds treated with our novel dressing showed significantly lower percentage of wound size and higher extent of collagen formation mainly due to the activity of sericin. We concluded that our novel bacterial nanocellulose incorporating PHMB and sericin was a safe and efficient wound dressing material for further investigation in the wound healing efficacy in clinic.

Keywords Bacterial nanocellulose · Sericin · PHMB · Wound dressing · Safety · Efficacy

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Introduction

Nowadays, a variety of wound dressings are developed for acceleration of wound healing by reducing wound infection and controlling the appropriate wound environment. However, some of them show adverse side effects such as pain, skin discoloration and renal toxicity [8]. Biosynthetic bacterial nanocellulose shows appealing characteristics to be applied as wound dressing. First, it has an ultrafine fiber structure with high capability to absorb and retain large amount of water (more than 200 times of its dry state), bringing the high capacity to absorb wound exudate and controlled moist environment over the wound [10]. Second, its nano-porous structure allows the easy transportation of active molecules or compounds to the wound. Third, the high tensile strength and flexibility make the bacterial nanocellulose to be a good barrier for wound protection. Fourth, it has cooling effect that can reduce pain [1]. Finally, the bacterial nanocellulose has high purity, non-toxicity, and does not activate allergic reaction [19].

In addition to the base bacterial nanocellulose wound dressing, active compounds incorporated in the dressing are important factors to accelerate wound healing. Either antimicrobial agent or accelerative wound healing agent are usually introduced to incorporate in the wound dressing. In terms of antimicrobial agent, polyhexamethylene biguanide (PHMB) is known as a broad spectrum antimicrobial agent having high antimicrobial activity against both Gram-negative and Gram-positive bacteria and low toxicity [22]. The PHMB-containing bacterial nanocellulose dressing has been reported to reduce biofilm and promote granulation tissue in wound without irritation [14]. In terms of accelerative wound healing agents, the high cost agents like growth factors, glycosaminoglycans, peptides, etc., are usually chosen, thus the resulting dressings are rather expensive so that most patients cannot afford for the treatment and loss the opportunity to use the advanced developing products. Sericin is a water-soluble protein extracted from silk cocoons. In the past, sericin was considered as a waste material in the textile industry. Recently, various biological activities of sericin such as antioxidation, bioadhesion, and activation of cell proliferation, collagen production and epithelialization in wounds have been recognized [4, 6, 7, 9, 12]. Thus, sericin is a biologically and cost effective accelerative wound healing compound for this purpose.

In fact, there is rarely that both antimicrobial and accelerative wound healing compounds are incorporated in one dressing. Therefore, we have attempted to develop an alternative wound dressing which composed of both PHMB as an antimicrobial agent and sericin as an accelerative wound healing component. The bacterial nanocellulose incorporating both PHMB and sericin has been introduced as novel wound dressing in our recent work [20]. The interaction, loading sequence, and loading concentration of PHMB and sericin were investigated to ensure that the wound dressing could provide both antimicrobial activity and promote collagen production during healing. We found that, in term of loading sequence, sericin needed to be loaded before PHMB to avoid any negative impacts. The optimal concentrations of the loaded sericin and PHMB to obtain the wound dressing with the most effective antimicrobial activity and enhanced collagen production were also reported [20]. In this study, the further in vitro, in vivo, and clinical safety of this novel wound dressing were performed. The in vitro cytotoxicity and cell migration tests, in vivo subcutaneous implantation and full-thickness wound healing tests in Wistar rats, and the clinical patch test in healthy volunteers were carried out to evaluate the biocompatibility, safety, and applicability of our novel bacterial nanocellulose incorporating both PHMB and sericin.

Materials and methods

Materials

Acetic acid (CH_3COOH), sodium hydroxide (NaOH), ammonium phosphate [$(NH_4)_3PO_4$], and other chemicals were of analytical grade purchased from Sigma-Aldrich, USA. Silk sericin solution was prepared from *Bombyx mori* cocoons supplied by Chul Thai Silk Co., Ltd. (Petchaboon province, Thailand) using a high temperature and high pressure degumming method [6]. Polyhexamethylene biguanide (PHMB) was kindly provided by Lonza Group Ltd. (Basel, Switzerland). Glycerin was an analytical grade from Ajax Finechem, Australia.

Preparation of bacterial nanocellulose dressing

Bacterial nanocellulose was produced by static culture of *Acetobacter xylinum* strain (Kasetsart University, Bangkok, Thailand) in coconut water medium according to the method of Verschuren et al. with a slight modification [29]. Briefly, 50 g of sucrose, 5 g of ammonium phosphate [$(NH_4)_3PO_4$], and 10 mL of acetic acid (CH_3COOH) was added into 1 L of boiled coconut water. The pH of solution was adjusted to 4.5 using acetic acid. Then, 10 mL of *A. xylinum* was added into the solution and the mixture was transferred to molds for fermentation. This mixture was incubated under sterilized static conditions at 30 °C for 10–12 days to form bacterial nanocellulose. Then, the bacterial nanocellulose was washed with 2 %w/v aqueous sodium hydroxide (NaOH) solution at 70 °C until a neutral pH was achieved. After that, 4 mL of 1 %w/v silk sericin was loaded on the bacterial nanocellulose ($10 \times 10 \times 0.01$ cm^3) by physical absorption. After silk sericin was absorbed, 10 mL of 0.3 %w/v PHMB was loaded on the silk sericin-containing bacterial nanocellulose. Finally, the PHMB/silk sericin-containing bacterial nanocellulose was immersed in 6 mL of 60 %w/v glycerin. The bacterial nanocellulose dressing containing PHMB and silk sericin, named as dressing A, was obtained. Bactigras® (0.5 % chlorhexidine acetate in white soft paraffin, Smith & Nephew Co., Ltd, London, UK), a commercially available wound dressing, was used as control dressings, named as dressing B.

In vitro cytotoxicity and migration tests

L929 mouse fibroblast cells (Chinese Academy of Preventive Medical Sciences, Beijing, China) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10 % fetal bovine serum (FBS) and antibiotics (100 U penicillin and 100 U streptomycin per mL) under 5 % CO_2 at 37 °C. The medium was changed every 2 days.

For cytotoxicity test, each of the sterilized dressings A and B (1×1 cm^2) was soaked in 3 mL of DMEM for 24 h. The extracted solutions of dressing were diluted with DMEM to 10, 20, 30, and 40 times (1/10, 1/20, 1/30, and 1/40). On the other hand, the cells (5×10^4 cells/well) were seeded in six-well plate and cultured for 24 h to allow cell attachment. Then, the medium was replaced with the diluted extracted solution or control (DMEM and Zn) and cultured for further 24 h. The number of viable cells was determined using the conventional 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.

A scratch test (injury to the cell monolayer) was performed following the method reported previously [24] to evaluate cell migration representing the expansion of a cell population on the surface. A six-well plate which contained 5×10^4 cells/well in DMEM and 10 % FBS was cultured to form a cell monolayer. After that, a linear scratch was generated in the monolayer using a sterile pipette tip (six points/scratch and two scratches/well). The cellular debris was removed by washing with phosphate buffer saline (PBS). Then, the extracted solutions of each dressing (1/15 dilution) and DMEM (control) were added to the wells and incubated at 37 °C with 5 % CO_2. After 24, 48, and 72 h, the photos of cells were taken under microscope (10×, Olympus CK2, Japan) and the distance of cell migrated was measured using Image J 1.42q/Java 1.6.0.10 program. The percentages of cell migration were calculated as follows:

$$\text{Percentage of cell migration } (\%) = (D_t - D_0)/D_0 \times 100, \quad (1)$$

where D_0 and D_t represent the initial distance between scratches and average distance between scratches at each time point, respectively ($n = 4$).

A Boyden's chamber assay was carried out to evaluate the chemotactic activity of L929 cells towards the medium containing dressings A and B [3]. Briefly, L929 cells were seeded in the upper chamber (5×10^4 cells/well in 100 µL) of 24-well transwell culture plates with 8-µm pore size-polycarbonate filter (Corning/Fisher Scientific, Schwerte, Germany). The extracted solutions of each dressing (1/15 dilution) were added into the lower chamber. The DMEM without dressing was used as a control. Cells were incubated at 37 °C, 5 % CO_2 for 24 h, then the migration of cells was evaluated. The migrated cells were fixed with 4 % paraformaldehyde for 30 min, washed with PBS, stained with trypan blue, and counted using light microscope at high magnification field (20×).

In vivo safety test

The animal experiments were performed according to Chulalongkorn University Animal Care and Use Committee (CUACUC 13/57) under standard sterile conditions. The experimental protocol was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Briefly, female Wistar rats (8 weeks old, weight ~200–300 g) were anesthetized, shaved the hair, and disinfected with 70 % ethyl alcohol. A 1 cm skin incision was made to form pockets in the subcutaneous tissue. Then, the dressing sample was inserted into each pocket. The wound was sutured with 6-0 prolene and disinfected with povidone iodine topical antiseptic solution. After 7, 14, 21, and 28 days of implantation, the rats were killed. The samples and surrounding tissue were retrieved, fixed with 10 vol% formalin solution, and embedded in paraffin. The paraffin-embedded samples were sectioned (4 µm) and immunohistochemical staining was proceeded as follows. The sections were deparaffinized in xylene and rehydrated prior to immunohistochemical staining. Endogenous peroxidase of the sample sections was quenched with 1 %v/v hydrogen peroxide in methanol, washed with 0.2 %v/v Tween in phosphate-buffered saline (0.2 % T-PBS), and blocked with 100 % FBS for 40 min. The sections were then incubated with mouse monoclonal anti FOXP3$^+$ antibody (Santa Cruz, USA) diluted in PBS + 1 %v/v FBS (Vector, USA, S1000) for 60 min, washed with 0.2 % T-PBS or three times, and incubated with labeled polymer HRP antimouse/rabbit EnVision kit (Dako, Denmark, K5007) for 60 min according to the manufacturer's instruction. For visualization, the sections were stained with diaminobenzidine staining (DAB, DAKO, Denmark, K3468) for 3 min, washed, counterstained with hematoxylin, and permanently mounted with DPX. Inflammatory responses to the dressings implanted including foreign body giant cells, fatty infiltration, fibrosis, and degranulation of mast cells were evaluated.

Clinical safety test in healthy volunteers

The safety test protocol of the dressings in healthy volunteers was approved by the ethic review committee for research involving human research subjects, Health Sciences group, Chulalongkorn University (COA No. 127/2557). It is a prospective, single-blinded, controlled study between October 2014 and February 2015 at the Department of Pharmacy Practice, Pharmaceutical Sciences, Chulalongkorn University. One hundred and five eligible healthy volunteers (18–65 years old) who could follow the instruction were recruited in this study. The exclusion criteria were the volunteers who used antihistamine and anti-inflammatory medications or any patches within 2 weeks before and during evaluation and were allergic to silk sericin and PHMB [24]. They would receive all information and signed consent form before starting. The demographic data of all volunteers, as presented in Table 1, were recorded at first time. The study was

Table 1 Demographic data of healthy volunteers for patch test

Document	Volunteer	%
Subjects	105	100.00
Sex		
Male	22	21.00
Female	83	79.00
Average age (years ± SD)	26.60 ± 10.83	
Average body mass index (kg/m^2 ± SD)	21.18 ± 3.12	
Occupation		
Government officer	3	2.86
State enterprise officer	2	1.90
Employee	31	29.51
Students	69	65.71
Underlying disease		
No	102	97.14
Hypertension	2	1.90
Diabetes mellitus	1	0.95
Current alcohol drinking	5	4.76
Current smoking	1	0.95

composed of five visits. Visits 1–3 were called as induction phase while visits 4 and 5 were called as re-challenge phase. At the first visit, the skins were randomly patched with the dressing A and B (2 × 2 cm^2). After 3 days (the second visit), the dressings were changed. The new dressings were patched onto the same area as the first visit. After another 3 days (the third visit), the dressings were removed. After that, there was a free period around 7–10 days. At the fourth visit, the skins were patched with the dressings on the same area as the first visit. After 3 days, at last visit, the dressings were removed. Both redness and darkness (erythema and melanin level) on skin of healthy volunteers were evaluated using Cutometer® with Mexameter mode (Courage + Khazaka electronic GmbH, Germany), and the photos were taken within 30 min after dressings were removed. The assessment in edema, papule, vesicle, and bullae has been performed by three dermatologists in every visit.

In vivo efficacy test

The experimental protocol of efficacy test was approved by Mahidol University Animal Care and Use Committee (MU-ACUC) in 2014. Twenty-four Wistar rats (8 weeks old, weight 300 ± 20 g) were anesthetized, shaved the hair, and disinfected with 70 % ethyl alcohol. Then, the full thickness wound (1.5 × 1.5 cm^2) was created on skin dorsal to subcutaneous depth. One rat received two wounds on left and right sides. The wounds were randomly covered with dressing A and B. All wounds were covered with transparent dressing (Tegaderm™) as a secondary dressing and adherent wrap (Coban™) as a tertiary dressing. The rats received 0.5–1.0 mg/kg of Tramadol via subcutaneous injection every day for 3 days. At days 3, 7, 14 and 21, wound size was evaluated by Visitrak™ digital device and wound infection was assessed by swab test [26]. Histomorphometric study was carried out to measure collagen formation in the wound at each period of post wounding. At days 3, 7, 14 and 21, six rats were euthanized and their wounded skins were removed and fixed in 10 % neutral buffered formalin. Then, the fixed tissues were processed following the standard tissue procedure. All tissue sections (4 μm) were stained with Masson's trichrome. From each section, ten fields (at 400×) were randomly examined and color images of 640 × 480 pixel resolution were acquired with a light microscope (BX51, Olympus®) and digital camera (DP20, Olympus®). Area fraction of collagen was semi-quantitative measured from those acquired images using ImageJ program, NIH. Briefly, color images were transformed to gray scale and located collagen bundle as interested area. Thus, the area fraction of positive collagen was determined as the percentage/image.

Statistical analysis

Statistical analysis among sample groups was evaluated using SPSS version 17.0 (SPSS Co., Ltd., Bangkok, Thailand). For cytotoxicity and migration tests, one way analysis of variance (ANOVA) was performed. Mann–Whitney U test was used to calculate statistical difference of wound size. The difference in area fraction of collagen and FOXP3$^+$ was analyzed by two way ANOVA. Repeated measure ANOVA was performed for erythema and melanin levels. The significant difference in edema and papule was analyzed by Kruskal–Wallis test and Chi-square test, respectively. The statistical difference among the sample groups was considered when $p < 0.05$.

Results and discussion

In vitro biological properties of the dressings

The in vitro biological properties of our novel dressings in terms of cytotoxicity and the promotion of cells migration were elucidated. Figure 1 presents the number of L929 cells cultured in the various dilutions of medium extracted solution of our novel dressing A, compared to that of the commercially available dressing B, DMEM, and Zn controls. The number of viable cells when cultured in the dressing A's extracted solution at all dilutions was comparable to that of the cells cultured in the dressing B's extracted solution and DMEM. This indicated that our novel dressing A was not toxic to the cells.

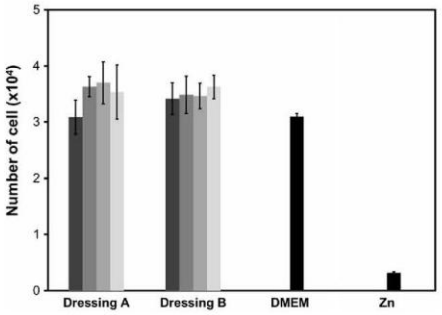


Fig. 1 Number of L929 cells after cultured in the various dilutions (1/10 *black bars*, 1/20 *dark grey bars*, 1/30 *grey bars*, 1/40 light *grey bars*) of medium extracted solution of the dressings *A* and *B* for 24 h. The cells cultured in DMEM and Zn (*black bars*) were served as negative and positive controls, respectively

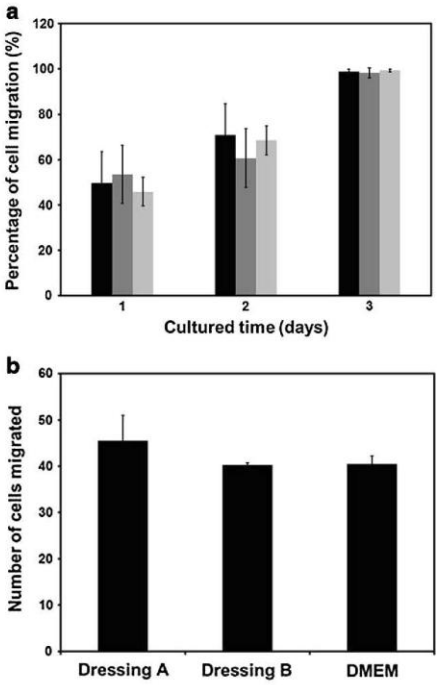


Fig. 2 **a** Percentage of L929 cell migration (scratch assay) when cultured in the undiluted medium extracted solution of the dressings *A* (*black bars*) and *B* (*grey bars*) and the DMEM control (*light grey bars*) for 1, 2, and 3 days. **b** Number of L929 cells migration towards the medium containing dressings *A* and *B* (Boyden's chamber assay). DMEM without the dressing was served as a control

Cell migration is also crucial for the wound healing. The cells with high ability of migration would accelerate the wound closure. Figure 2a shows the percentage of L929 cell migration when cultured in the medium extracted solution of the dressing A, compared to that of the dressing B and DMEM control, which was evaluated from the scratch assay. No significant difference in the cell migration percentage among all sample groups. In addition, the chemotactic activity of L929 cells migrated towards the medium containing the dressings, evaluated from Boyden's chamber assay, is shown in Fig. 2b. There was also no difference in the number of cell migration among all sample groups. The dressing A, possibly due to the sericin released, seemed to promote the migration of L929 cells as good as the commercially available dressing B and the DMEM control. The promotion of cell migration by sericin was described in our previous work [13]. These in vitro data confirmed the biological properties of our novel dressing A to support wound healing.

In vivo safety of the dressings

The in vivo subcutaneously implantation and full-thickness wound models were performed with the dressing A to confirm its safety and wound healing efficacy prior to the clinical trial. Figure 3a shows the histopathological changes in tissue when the dressings A and B were implanted for 14 days. Severe inflammatory response as characterized by highly infiltration of foreign body giant cells, fatty, and fibrosis was found throughout the commercially available dressing B implanted. The implantation of the novel dressing A showed the lower inflammatory response and the inflammation was limited to the tissue interim of the dressing. The percentage of mast cell degranulation in the dressings implanted which indicated the extent of inflammatory reaction was presented in Fig. 3b. The percentage of mast cell degranulation in the commercially available dressing B was significantly higher than that of dressing A along the implantation period. Noticeably, the mast cell degranulation in the dressing A was not observed after 21 days of implantation. For the degranulated mast cells, the granules were released from the cytoplasm due to the rupture of cell membrane. This could activate neutrophil and histamine and led to acute inflammatory.

Furthermore, the immunohistochemical staining of monoclonal mouse anti FOXP3$^+$ of the dressings implanted for 28 days was shown in Fig. 4a. The transcription factor FOXP3$^+$ is known to control the development and function of natural and adaptive T regulatory cells (Tregs)

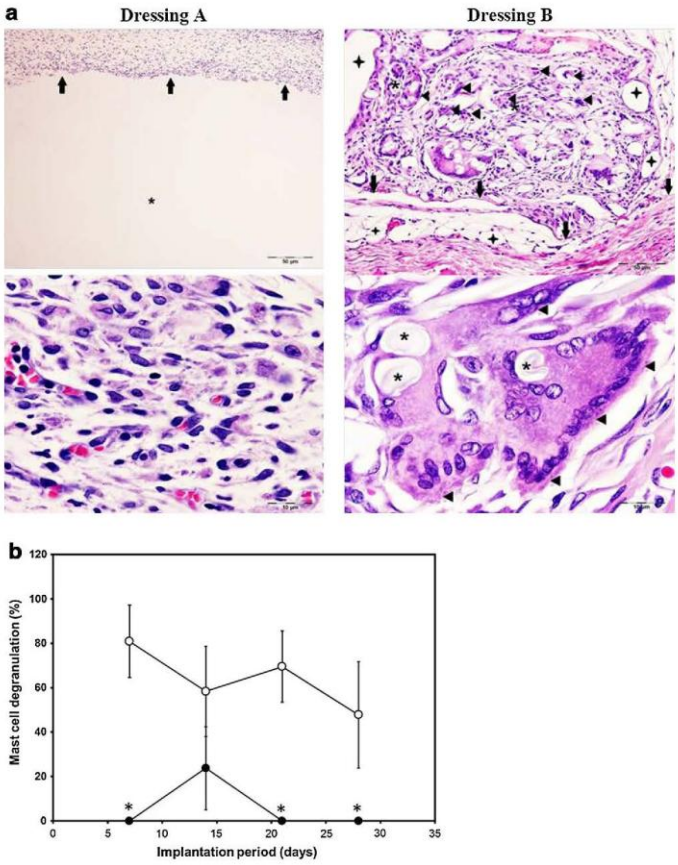


Fig. 3 **a** Histopathological changes on tissue when the dressings *A* and *B* were implanted for 14 days (*left-pointing triangle* diffused accumulation of numerous, *diamond* foreign body giant cells, *four-pointed star* fatty infiltration, *asterisks* implanted materials, *upward black arrows* tissue interface). **b** Percentage of mast cell degranulation in dressings *A* (*closed circle*) and *B* (*open circle*) implanted for 7, 14, 21, and 28 days. *Significant difference when compared with the value of dressing *B* at corresponding implantation period ($p < 0.05$)

which can promote peripheral self-tolerance by suppressing excessive immune responses [23]. Several studies demonstrated that tolerogenic dendritic cells enhance FOXP3$^+$ Tregs development [16, 25, 27, 30]. Mutation or lack of FOXP3$^+$ Tregs is a cause of autoimmune disease, inflammation and allergy [23]. Therefore, it is a widely used marker for in vivo allergic evaluation. Herein, the higher number of Tregs was clearly seen in the dressing A than the dressing B. Figure 4b presented the *H* score of FOXP3$^+$ Tregs in both dressings after implantation for 7, 14, 21, and 28 days. The *H* score of FOXP3$^+$ Tregs of dressing A showed increasing trend and it was significantly higher than that of dressing B along the implantation period. The FOXP3$^+$ Tregs also had positive correlation to polymorphoneuclear cells or neutrophil infiltration to the tissue interface. This was in agreement with the study of Tateosian et al. that elastase enzyme in neutrophil can enhance tolerogenic dendritic cell to generate CD4$^+$ FOXP3$^+$ Tregs [28]. The more expression of FOXP3$^+$ related with hypo-responsiveness of activated T cell which led to low inflammatory response [31]. Lin et al. also found that FOXP3 mutation in mice can be a cause of allergic inflammation response and uncontrolled T cell action [15]. Moreover, adoptive transfer of CD4$^+$CD25$^+$FOXP3$^+$ Tregs from healthy to disease mice could improve allergy, thus the FOXP3$^+$ Tregs were important factor in the allergic regulation [21]. In this study, we found that FOXP3$^+$ Tregs had negative correlation to giant cell

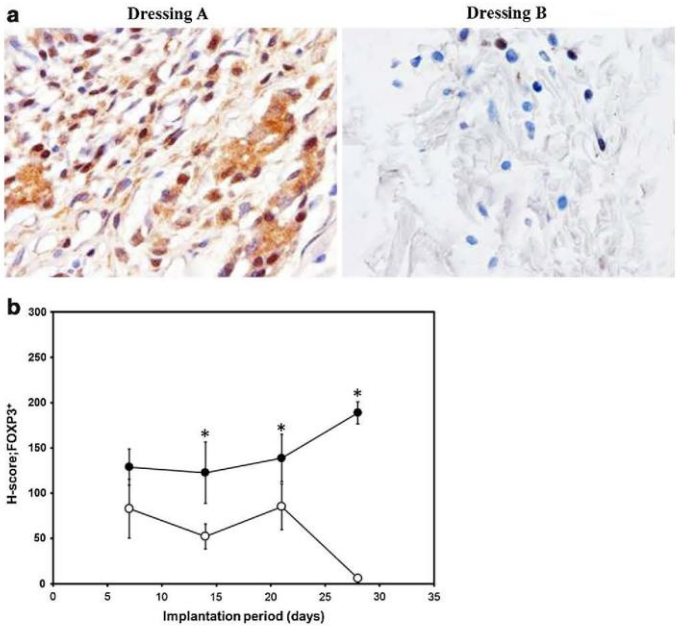


Fig. 4 **a** Immunohistochemical staining of monoclonal mouse anti FOXP3$^+$ of the dressings *A* and *B* implanted for 28 days. **b** *H* score of FOXP3$^+$ Tregs of the dressings *A* (*closed circle*) and *B* (*open circle*) implanted for 7, 14, 21, and 28 days. *Significant difference when compared with the value of dressing *B* at corresponding implantation period ($p < 0.05$)

infiltration and mast cell degranulation which were both characterized as high immunogenic inflammatory responses and severity of pathological lesions. In overall, our novel dressing A showed lower inflammatory response and allergic reaction than the commercially available dressing B. This could be that the antimicrobial PHMB component of the dressing A reduced the risk of infection and inflammatory reaction during the implantation. The PHMB-containing dressing was shown to decrease the bacteria in wound and prevent wound infection [18]. Eberlein et al. also reported that PHMB-containing bacterial nanocellulose dressing potentially reduced the local wound infection better than the silver dressing [11]. Moreover, sericin and bacterial nanocellulose components of the dressing A have been shown to induce minimal inflammatory reaction [2, 7, 24].

Clinical safety of the dressings

After the in vivo safety and efficacy of the dressing A were confirmed, its safety for the clinical use was tested in the normal skin of healthy volunteers using a patch test. The results were reported as a erythema level represented redness and a melanin level represented darkness of the skin patched with the dressings, as shown in Fig. 5. From the total 105 healthy volunteers of this study, the levels of erythema and melanin were not different for every visit. These levels were closed to the levels of healthy skin, as reported by Maenthaisong et al. [17] (~220 of erythema and ~230 of melanin). Furthermore, the assessment data from three dermatologists demonstrated that most of the patched skins did not show edema (more than 98 %) and papule (more than 97 %). No vesicle and bullae was observed on the skins after patched with either dressing A or B. Therefore, it could be concluded that the bacterial nanocellulose incorporating PHMB and sericin (dressing A) was non-irritant and safe for the further evaluation of clinical wound healing efficacy.

In vivo wound healing efficacy of the dressings

The in vivo wound healing efficacy of the dressings was tested in a full-thickness wound in rats. After the created full-thickness wounds were treated with the dressings A and B for 3, 7, 14, and 21 days, the percentage of wound size was measured as shown in Fig. 6a. At 3 and 7 days after treatment, the percentage of wound size treated with both dressings was not different (~85 % at 3 days and

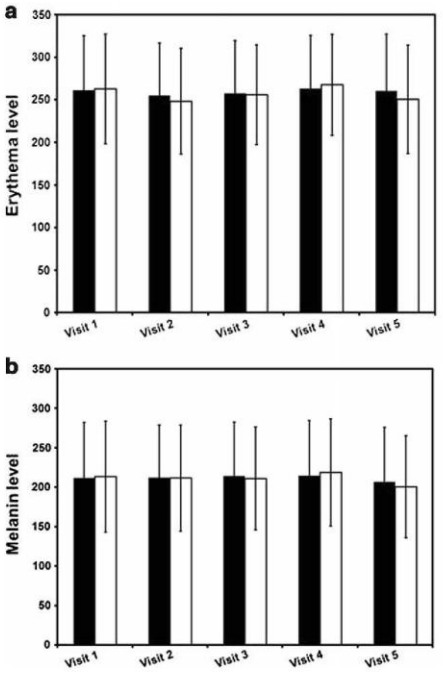
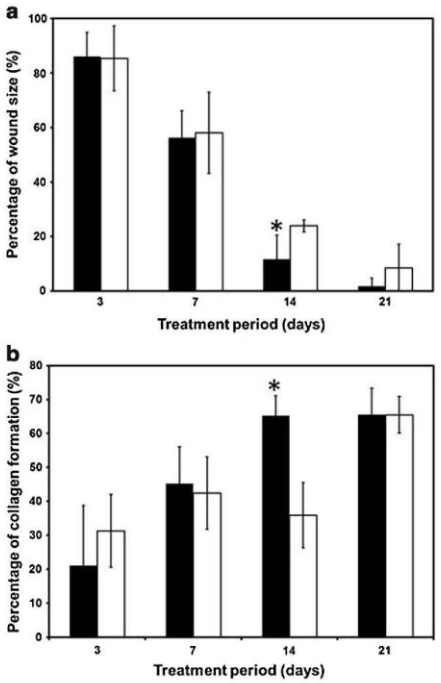
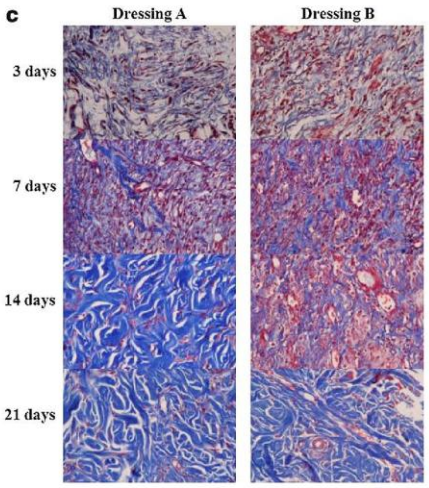


Fig. 5 **a** Erythema and **b** melanin levels of the skin of healthy volunteers patched with the dressings A (*closed square*) and B (*open square*) at different visits





~60 % at 7 days). The percentage of wound size treated with the dressing A tended to be lower than that of dressing B from day 14. Furthermore, at 21 days after treatment, the percentage of wound size treated with dressing A was close to zero, indicating that the wound was almost closed completely. This was agreed with the results of previous study reported that sericin cream could reduce wound size in rats without allergic reaction and causing of inflammation [5, 7].

Collagen synthesized by fibroblast cells is one of key indicators of wound healing efficacy. Figure 6b, c demonstrates the extent of collagen formation in the wounds treated with dressings A and B for 3, 7, 14, and 21 days. The extent of collagen formation in both wounds was increased with time. The difference in collagen formation in both wounds was not observed for the first week of treatment. However, at 14 days of treatment, the formation of collagen in the wound treated with the novel dressing A reached about 65 % which was significantly

◀**Fig. 6** **a** Percentage of wounds size and **b** collagen formation in the wounds after treating with the dressings A (*closed square*) and B (*open square*) for 3, 7, 14, and 21 days. *Significant difference when compared with the value of dressing B at corresponding implantation period ($p < 0.05$). **c** The area fraction of collagen formed in the wounds after treated with the dressings A and B for 3, 7, 14, and 21 days

higher than that of the wound treated with dressing B (~36 %). It has been reported that the optimum concentration of sericin could induce the production of type I collagen in wound [6]. Therefore, we supposed that the sericin in our novel dressing A was a potential component to promote collagen production and wound healing. The bacterial nanocellulose may also provide the appropriate environment that supported the wound healing. Furthermore, the reduced inflammation responses of the wound treated with the dressing A as described previously might accelerate the subsequently wound healing cascades.

Conclusions

The novel bacterial nanocellulose incorporating sericin and PHMB was developed to be applied as a wound dressing material. For this purpose, the biological properties of the novel dressing were tested in vitro, in vivo, and clinic in comparison to the commercially available dressing. The in vitro data demonstrated that our novel dressing was not toxic and promoted the migration of L929 mouse fibroblast cells as good as the commercially available dressing. The in vivo results confirmed the safety and wound healing efficacy of the novel dressing as characterized by the low inflammation response and potentially reduced wound size with high extent of collagen formation. Finally, we showed that our novel dressing did not irritate the skin of healthy volunteers. The wound healing efficacy of this novel dressing in clinical trial has been investigated. It is expected that this novel wound dressing would have antimicrobial property to prevent the wound infection due to the PHMB activity and have accelerated wound healing property due to the sericin activity while the bacterial nanocellulose based dressing would preserve other properties to be suitable for wound healing.

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REVIEW ARTICLE

Effect of animal products and extracts on wound healing promotion in topical applications: a review

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ABSTRACT

Wound healing is a natural process of body reaction to repair itself after injury. Nonetheless, many internal and external factors such as aging, comorbidity, stress, smoking, alcohol drinking, infections, malnutrition, or wound environment significantly affect the quality and speed of wound healing. The unsuitable conditions may delay wound healing process and cause chronic wound or scar formation. Therefore, many researches have attempted to search for agents that can accelerate wound healing with safety and biocompatibility to human body. Widely studied wound healing agents are those derived from either natural sources including plants and animals or chemical synthesis. The natural products seem to be safer and more biocompatible to human tissue. This review paper demonstrated various kinds of the animal-derived products including chitosan, collagen, honey, anabolic steroids, silk sericin, peptides, and proteoglycan in term of mechanisms of action, advantages, and disadvantages when applied as wound healing accelerator. The benefits of these animal-derived products are wound healing promotion, anti-inflammatory, antimicrobial activity, moisturizing effect, biocompatibility, and safety. However, the drawbacks such as allergy, low stability, batch-to-batch variability, and high extraction and purification costs could not be avoided in some products.

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1. Wound healing process

Wound healing is a natural process in which a body response after injury to protect itself from outer environments while the repairing is occurred simultaneously. The process composes of three major phases which are inflammation, proliferation and remodeling [1]. After skin is injured, the wound healing process immediately begins with the formation of clotting at the wound site by vasoconstriction and platelet aggregation. The pro-inflammatory cytokines and chemokines including interleukin (IL-1), and tumor necrosis factor (TNF-α, TNF-β) are released to activate inflammatory cells [2]. Neutrophil, macrophage, and fibroblasts are infiltrated to the wound site. Then, nitric oxide, oxygen free radicals, and

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matrix metalloproteinase (MMP) are generated to prepare for next proliferation phase [3]. The proliferation phase involves epithelialization and angiogenesis in which transforming growth factors (TGF) and epidermal growth factor (EGF) are important factors to stimulate proliferation, migration, and differentiation of fibroblasts and keratinocytes [4]. Type I collagen is produced by fibroblasts to form the neotissue. Subsequently, in remodeling phase, the newly formed collagen is rearranged into tissue while keratinocytes migrate from the edge to close the wound site. Moreover, transforming growth factors beta (TGF-β) plays an important role in collagen matrix remodeling and inhibiting MMP production to control an appropriate healing environment [5].

It has been recognized that many internal and external factors such as aging, comorbidity, stress, smoking, alcohol drinking, infections, malnutrition, or wound environment had significantly effect on the quality and speed of wound healing. The unsuitable conditions may delay wound healing process and cause chronic wound or scar formation [6]. Many researches have attempted to search for wound healing agents that can accelerate wound healing process with safe and biocompatibility to human body. Among these, the products derived from animals have been widely investigated because they usually have biological activities that support wound healing and show minimal toxicity.

2. Wound healing accelerators derived from animals

In this study, the wound healing accelerators derived from animals including chitosan, collagen, honey, anabolic steroids, silk sericin, peptides, and proteoglycan were reviewed and compared in term of mechanisms of action, advantages, and disadvantages when applied in topical applications.

2.1. Chitosan

Chitosan is a deacetylated derivative of chitin which is mostly found in exoskeletons of arthropods including shrimp, crab, and insects [7]. The major sources of chitin for commercial products are crab and shrimp shells, which are the most abundant waste in food production [8]. There are alpha, beta, and gamma forms of chitin. Alpha chitins are composed of two antiparallel molecules per unit cell with inter-sheet hydrogen bonds in crystal structure while beta chitins are composed of one parallel molecule per unit cell without inter-sheet hydrogen bonds in crystal structure. Gamma chitins are composed of two antiparallel chains alternating with one parallel strand. The differences in characteristics of alpha, beta, and gamma chitin are summarized in Table 1. Chitosan is a polysaccharide composes of β (1 → 4) glucosamine linked N-acetyl-D-glucosamine. It can have molecular weight between 50 and 2000 kDa. Physical and chemical properties of chitosan depend on degree of acetylation and acetyl group [9]. Chitosan is soluble in acidic solution and insoluble in high pH environment because amine group is deprotonated and lost its charge [8]. It is biodegradable; the low degree of deacetylation or low molecular weight is degraded more rapidly [8]. Chitosan is also biocompatible and low toxic. It was reported that the glucosamine units of chitosan are an effective wound healing accelerator [10]. Therefore, various forms of chitosan were widely used for wound healing including powders, gels, films, fibers, and scaffolds. The commercial products of chitosan and derivative for wound healing are Tegasorb®, Tegasderm®, HemCon Bandage™, Chitodin®, and Trauma Dex® [11].

Table 1. Characteristics of α-Chitin, β-Chitin, and γ-Chitin [9,164].

Chitin	Source	Molecular arrangement	Reactivity for chemical and enzymatic transformation	Thermal degradation	Solubility in common solvent	Chitosan viscosity [165]	Chitosan Molar Mass [165]
α -Chitin	Crab tendons Shrimp shells Lobster Krill Insect cuticle Fungal and yeast cell wall	Two antiparallel molecule per unit cell with inter and intra-sheet hydrogen bonds in crystal structure	+	+	+	+++	+++
β -Chitin	Squid pens Pogonophoran tubes Vestimetiferan worms tubes	One parallel molecule per unit cell with intra but without inter-sheet hydrogen bonds in crystal structure	+++	+++	+	+	+
γ-Chitin	Cocoon fibers of the *Ptinus* beetle Stomach of *Loligo* squid	Two antiparallel molecule and one parallel molecule per unit with inter and intra-sheet hydrogen bonds in crystal structure (like α –Chitin than β –Chitin)	++	++	+	++	++

Note: (+, ++, +++ indicate the extent of activity from low to high).

2.1.1. Processing methods

For chitosan extraction, there are two major methods which are chemical deacetylation and enzymatic deacetylation [9]. For chemical deacetylation process, chitin is treated in concentrated sodium hydroxide to produce chitosan. It is widely used process due to the low cost. However, it consumes a lot of energy and produces a lot of waste. Moreover, temperature, time, and solvent concentration have effects on chitosan characteristics. For enzymatic deacetylation process, the chitin deacetylase enzyme is used to produce chitosan by N-acetamino bonds hydrolysis. Enzymatic deacetylation by fungi may be an alternative process. Chitosan may contain bacterial and metal contamination because it is produced from exoskeletons of arthropods. However, the extraction process including deproteinization and demineralization step can remove many contaminations [12,13]. For biomedical grade chitosan product, further purification step including endotoxin removal and sterilization should be added. Filter sterilization is the most simple method that can remove bacterial contamination and did not have an effect on stability of chitosan products. Moreover, ethylene oxide sterilization seems to be the most appropriate for chitosan materials because it has less effect on chemical and physical properties of chitosan comparing with other methods such as autoclaving, dry heat, gamma irradiation, and ultraviolet light radiation [14]. It is reported that the endotoxin in chitosan wound dressing was less than collagen and did not induce pyrogenicity in rabbits [15].

2.1.2. Mechanisms on wound healing

Chitosan promotes wound healing through two major pathways. Firstly, the N-acetyl-D-glucosamine unit of chitosan initiates fibroblast proliferation and collagen production [10]. Its positive charge of chitosan has an electrostatic interaction with glucoaminoglycans, leading to growth factor attraction [16]. Secondly, macrophages are activated by N-acetyl-D-glucosamine lead to phagocytosis, and the release of mediators including TGF-β1 and platelet derived growth factor. These biological mediators subsequently accelerate extracellular matrix (ECM) synthesis [17]. Moreover, chitosan activates the production of IL-1 that controls the fibroblast proliferation and collagen synthesis [18]. It is also reported that chitosan stimulates the release of IL-8 by fibroblasts which leads to angiogenesis and migration of neutrophils. The blood vessel was found in the granulation tissue in wound treated with chitosan after 17 day of treatment [19,20]. Chitosan can act as a hemostat that supports in blood clotting through fibrinogen and plasma protein attraction. It contained amine group that can bind with negative charge on red blood cell membrane leads to hemaggulation [21]. Moreover, it can block nerve ending resulting in pain reduction [11]. Therefore, it helps in faster wound healing and scar prevention.

The previous study applied chitosan cotton fiber for the treatment of open dorsal skin wound of beagles. They found that granulation tissue of full thickness layer was significantly elevated in chitosan-treated wound after 6, 9 and 15 incision day comparing with the bandage without chitosan (control group). The results of this study showed that chitosan could accelerate the proliferation of fibroblasts and the production of type III collagen [22]. Chitosan sponge dressing and hydrogel were shown to decrease wound healing time and wound size of dorsal wound in rabbits [23] and rats together with significant increasing of granulation tissue formation and epithelialization [20]. For the treatment of second degree burn wound in rats, epithelialization rate of the wound treated with chitosan gel incorporating EGF (10 μg/ml) was significant higher than that of the EGF gel-treated group [24].

Chitosan nanofiber mats showed the faster wound size reduction than gauze dressing at first week after tissue damage [25]. In addition, the molecular weight of chitosan has significant effect on wound healing. The burn wounds treated with high molecular weight chitosan (MW = 2,000,000) topical gel showed the highest extent of epithelial and granulation tissue, and fastest wound closure when compared with that treated with the low molecular weight chitosan (MW = 70,000) gel and gel without chitosan [26]. In skin graft donor site patients' treatment, chitosan membrane also showed positive effect on the re-epithelialization and regeneration of the granulation layer than the control (Bactigras®; chlorhexidine paraffin gauze dressing) [27].

2.1.3. Antibacterial effect

The chitosan structure is composed of amine groups (cationic polymers) which can strongly bind to the anionic proteins in cytoplasmic membrane of bacterial cells, leading to imbalance of cell membrane and cell death [11]. Moreover, low molecular weight chitosan (less than 5 kDa) could penetrate cell wall of bacteria, combined with DNA, and inhibiting mRNA and DNA transcription [28]. The differences in antibacterial mechanisms of chitosan against gram positive and gram negative bacteria were reported. Zheng L-Y et al. found that antimicrobial activity of chitosan against gram positive bacteria (*Staphylococcus aureus; S. aureus*) increased when molecular weight is increased because the high molecular weight chitosan can form an external barrier to block nutritional intake. Nevertheless, antimicrobial activity of chitosan against gram negative bacteria (*Escherichia coli; E. coli*) increased when the molecular weight is decreased because the low molecular weight chitosan easily entered to bacterial cell [29]. Chitosan with low pH (pH < 6.5) and low degree of acetylation was also more rapidly absorbed to the bacterial cell wall and cation of chitosan was augmented [9,30].

For antimicrobial test, chitosan can inhibit either gram positive or gram negative bacteria. The inhibition zone of *S. aureus* was higher in the chitosan fabric dressing group (1% w/w) when compared with the non-chitosan dressing. Furthermore, the inhibition zone increased with the amount of chitosan applied [31]. In antimicrobial broth test, the optical density of *E. coli* in the chitosan with silk fibroin nanofiber membrane broth was lower (lower bacteria) than the non-chitosan membrane broth. However, this difference was not observed in *S. aureus* [32]. The chitosan/gelatin sponges were shown to have the higher antimicrobial activity against a gram positive (*Streptopcpccus*) than cefradine but less than ciprofioxacin and penicillin. Its antibacterial effect against gram negative bacteria (*E. coli* K88) was also better than penicillin but less than ciprofioxacin and cefradine [23].

2.1.4. Biological safety

Degree of deacetylation of chitosan has no significant influence to keratinocytes and fibroblasts cytocompatibility [33]. The chitosan dressing materials including gel, membrane, and sponge were proved to be safe *in vivo* and showed no erythema, irritation, and toxicity [20,23,34]. Chitosan sponge treated group had no irritation on rabbit skin which was comparable to the vaseline gauze treated group [23].

2.2. Collagen

Collagen is the protein which is the important component in human ECM of skin, bone, and other tissues. It can also be extracted from many kinds of animal including bovine

(cow), porcine (pig), or marine (fish, jellyfish, star fish, and squid). Table 2 demonstrates the type, properties, and limitations of collagen obtained from different sources. The basic structure of collagen is composes of three polypeptide chains formed into triple helix and has molecular weight around 300 kDa [35]. The amino acid sequence of collagen contains arginine-glycine-aspartic acid motif which is the specific cell adhesion domains that promote cell growth, differentiation, and activities [36]. Collagen is biocompatible and safe. However, it has a fast rate of biodegradation and low denaturation temperature. Some denatured collagen such as gelatin may lose wound healing property in the production [37].

2.2.1. Processing methods

Different processes are used to extract collagen depending on the source of collagen. The raw material collected from animals is preserved at −25 or −4 °C. Then, non-collagen was removed by sodium chloride, sodium hydroxide, and calcium hydroxide. After that, acetic acid or hydrochloric acid solution was used for demineralization process. The product was further digested by enzyme and acid to obtain the collagen [38]. Sterilization methods of collagen for using as biomaterial were studied. Ethylene oxide sterilization was the effective method in term of maintaining the physical and chemical stability of collagen products. Furthermore, the effectiveness of gamma and beta irradiation (up to max dose 20 kGy) sterilization was also reported with the collagen wound dressings [39,40]. Endotoxin in bovine collagen was less than porcine collagen dressing. Nevertheless, both of them did not induce pyrogenicity in rabbits [15].

2.2.2. Mechanisms on wound healing

Collagen plays a chemotactic role to attract skin fibroblast to the wound [41,42] and supports fibroblast activity to produce the granulation tissue. It can cross-link with fibronectin to generate appropriate surrounding environment for re-epithelialization process [43]. Moreover, collagen also binds platelet and generates thrombin in hemostasis [44]. It also activate macrophage and important factors to induce angiogenesis for wound healing process [37,45,46]. Collagen type I and III are reported as the important factors in wound healing process.

Normally, the native collagen may be destroyed by elevated level of MMPs, leading to non-healing wound [47]. Therefore, collagen dressing shows a superior property by delivery the substitute collagen to the wound and reduces elastase level in a wound environment [48]. The MMPs binding activity of substitute collagen also delayed non-healing wound process. The collagen supports chemotaxis action between fibroblast and native collagen to promote natural wound healing [37]. Moreover, collagen dressing can absorb wound exudate and provide the moist wound environment [47].

There are many studies about wound healing property of collagen. The gel and membrane made of bovine collagen were reported to accelerate wound healing by activating granulation in the wound bed [46,49]. The oxidized regenerated cellulose/collagen also promoted fibroblast proliferation and cell migration. *In vivo* test showed that the diabetic wound size of collagen treated group was statistically smaller than standard treatment (gauze dressing) [50]. Moreover, collagen-base dressing such as Promagan® dressing (55% collagen) also decrease wound area and painful [51–53] than conventional dressing such as Adaptic® non-adherent dressing or gauze dressing. However, some studies showed no significant result of collagen on wound healing. The ulcer closure percentage of bovine collagen gel treated group and standard moist dressing treated group were not

Table 2. Type, properties and limitations of collagen obtained from different sources.

		Properties							Cost
Collagen sources	Most collagen type	Quality for skin treatment	Cell binding property	Fibroblast viability when contact with collagen	Biocompatibility	Antigenicity	Denaturation temperature (Td)	Limitation	
Bovine (Achilles tendon)	I, III	++	++	++	++	+	37 °C	• Allergic report around 3% [38] • Concerning about Bovine spongiform encephalopathy (BSE) or Transmissible spongiform encephalopathy (TSE) [60] • Forbidden in some culture	+
Porcine (Skin)	I, III	++	++	++	++	+	37 °C	• Integration of porcine collagen into scar tissue [60] • Concerning about zoonosis contamination [38,166] • Forbidden in some religion and culture	+
Marine (Bone, skin, fin, scales)	I	+++	++	+++ [167]	++	+	18–30 °C [168]	• Cross reactivity among fish gelatin [169] • Lower yield obtained (12 g of collagen per1 kg of raw material [38] • Low denaturation temperature	++

Note: +, ++, +++ indicate the extent of activity from low to high.

significantly different after 12 weeks [54]. Graumlich et al. demonstrated that healing time and percentage of complete wound healing of pressure ulcer after 8 weeks between topical collagen treated wound and hydrocolloid treated wound were not statistically different (51% vs. 50%, respectively) [55]. Donaghue et al. studied in 75 diabetic foot ulcer. Forty-eight percent of the patients received the treatment with alginate dressing containing 90% collagen achieved complete healing while those treated with normal saline moistened gauze was only 36% [56]. The comparison between collagen dressing and conventional dressing (including silver sulfadiazine, nadifloxacin, povidone iodine, or honey) in burn and chronic wound treatment demonstrated that percentages of healing of both groups after 8 weeks were not different (87% vs. 80%, respectively). However, the conventional treatment group required more treatment of partial split thickness skin graft than the collagen dressing treated group [57].

2.2.3. Biological safety

In term of safety, adverse reactions of collagen to wound tissue were rarely found [55]. There were few cases reported about the IgE-mediated reaction of bovine collagen leading to allergic reaction. It was presented in conjunctival edema after highly purified bovine collagen contacted to the eye during ophthalmic surgery [58,59]. The bovine-derived type I collagen dressing showed the developed dermatitis three case from 20 case [49]. Collagen dressing may induce overgranulation [60]. In addition, the topical collagen products are expensive so that they may not be the best choice of wound dressing.

2.3. Honey

Honey is produced from nectar that is stored and transferred by bees to honeycomb. The enzyme secreted from the bee influences the physical and chemical characteristics of honey. The main compositions of honey are sugar and water. The major sugar components in the honey are fructose (38.2%) and glucose (31.3%) [61]. Moreover, it has other components including enzyme, amino acid, organic acid, carotenoids, vitamin, mineral, and aromatic substance [62]. These components are varied depending on botanical origins, geographic origins, storage conditions and temperature [63]. The composition of honey also has effects on its physical and chemical properties. For example, the water content in various type of honey lead to differentiate of viscosity, crystallization, color, flavor, specific gravity, and solubility [62]. Physical properties also change to darker color. Honey contains acidity pH around 3.2–4.5 [62]. It has a hygroscopic property to absorb water from environment. Because of 18.8% water content of honey, it can absorb moisture from environment humidity above 60% [64]. Various types of honey such as manuka honey, fynbos honey, and tualang honey obtained from different tree or nectar have been studied for wound treatment. Examples of clinical studies of topical honey treatment are shown in Table 3.

2.3.1. Processing methods

Honey for medical treatment has to be sterilized by gamma irradiation to kill bacterial spore [65]. However, gamma irradiation cannot eliminate endotoxin in honey. The low amount of endotoxin in natural honey (~56 to 690 pg/ml) does not induce inflammatory. Nonetheless, higher endotoxin concentration may induce inflammatory response [66].

Table 3. Clinical studies of topical honey treatment.

Study design	Wound type	Number of subjects	Honey type	Control treatment	Outcome	Side effects
Randomize control trial [170]	• Shallow wounds (deeper ≤ 2 cm, size ≤ 100 cm^2) • Abrasions wound (size 10 -100 cm^2)	87 (Sample 40, control 42, dropped out 5)	Natural honey	IntraSite gel® (amorphous hydrogel contain propylene glycol)	• Healing times of both groups were not significantly different • Satisfactions of both groups were not significantly different • Honey was low average cost of treatment per patient than IntraSite® Gel (R0.49 vs. R12.03)	Itching: Honey 27%, IntraSite gel® 31% Pain: Honey 10% Short time burning: Honey 2 case
Randomize control trial [171]	Partial-thickness burns of less than 40% of TBSA (Total body surface area)	100 (Sample 50, control 50)	Natural honey	Mafenide acetate	• Epithelialization of honey-treated group was significantly higher than the control (100% vs. 84%, respectively, after 21 days) • Amount of bacterial reduction of honey-treated group were higher than control (45 vs. 32, respectively, after 21 days)	No irritation, allergy, or other side effects in both groups
Randomize control trial [172]	First and second degree of burn of less than 50% of TBSA	78 (Sample 37, control 41)	Natural honey	Silver-sulfadiazine (SSD)	• Healing times of honey-treated group were less than the control (18.16 vs. 32.68 days, respectively) • Bacterial reduction rate of honey-treated group was faster than control • Percentage of complete healing wounds of honey-treated group were significantly higher than the control (81% vs. 37%, respectively)	No report
Randomize control trial [173]	Chronic wounds	42 (Sample 22, control 20)	Natural honey	Povidone-iodine dressing	• Percentage of complete healing wounds of honey-treated group were higher than the control (31.82% vs. 0%, respectively, after 6 weeks) • Wound surface area and pain score in honey-treated group were significantly less than the control group	Honey: no adverse skin reaction Povidone-iodine dressing: 2 adverse skin reaction
Randomize control trial [174]	Split-thickness skin graft donor site	100 (Sample 50, control 50)	Natural honey	Vaseline gauze	• Percentage of complete healing wounds of honey-treated group were significantly higher than the control (100% vs. 76%, respectively, after 10 days) • No bacterial growth was found in both groups • Pain in honey-treated group was not significantly less than the control group	No allergic reaction in both groups
Randomize control trial [175]	Neuropathic diabetic foot ulcers	63 (Sample 32, control 31)	Manuka honey	Saline-soaked gauze	• Healing times of honey-treated group were significantly less than the control group (31 ± 4 vs. 43 ± 3 days, respectively) • Number of patients required antibiotic treatment of honey-treated group was none but that of the control group was 29%	No report

2.3.2. Antibacterial effects

Honey is reported as bacteriostatic and bactericidal agent depending on its concentration. Low concentration of honey (4–8% v/v of honey and 5–11% v/v of manuka honey) showed the bacteriostatic property while the high concentration of honey (5–10% v/v of honey and 8–15% v/v of manuka honey) had the bactericidal property [64]. The honey contains high concentration of sugar, so it presents the high osmolality property. It draws fluid out of the environment to create an unsuitable environment for organism existence [65]. Low pH of honey (pH 3.2–4.5) created inappropriate environment for the growth of microorganism [64]. Another mechanism of honey for antibacterial activity is a hydrogen peroxide production. Hydrogen peroxide is a broad spectrum antimicrobial molecule that is produced by glucose oxidase enzyme from bees. The hydrogen peroxide produced from bees is not toxic to the cells due to its low concentration, which differ from the chemical synthesized hydrogen peroxide [67]. However, the osmolality property may not be sufficient when the honey is diluted or interacted with enzyme catalyze in wound exudate [68]. Therefore, bee's honey may not be suitable for the treatment of high exudate wound. On the other hand, manuka honey has a methylgloxal component as an antimicrobial agent that is unrelated with hydrogen peroxide production [69]. It also shows antibacterial effect against biofilm and various microorganisms such as Methicillin-resistant *S. aureus*, *S. aureus*, *Pseudomonas aeruginosa*, and *E. coli* [70]. In addition, honey is reported to reduce odor of the wound and has a debridement activity [65].

2.3.3. Anti-inflammation effects

The excessive inflammatory reaction can delay wound healing process. Honey can reduce the extent of inflammatory. Honey suppresses protease activity which is an important enzyme in inflammatory process. The acidic property of honey produces low pH environment that is not appropriate for protease activity [71]. It increases nitric oxide that plays an important role in angiogenesis and healing process. It also decreases prostaglandin in blood circulation and reduce inflammation and pain in wound [72]. Moreover, honey contains antioxidants including flavonoids, phenolics, and vitamin C that interact with reactive oxygen species and reduce the cellular damage [67,73].

2.3.4. Biological safety

Honey is non-toxic, non-allergic, and non-irritate to wound tissue [74]. However, there are some cautions of using. Patients who have pollen allergic history may be allergic to honey. Furthermore, dehydration of tissue may be found in the extensive honey treatment. Some patients may be sensitive to acidity of honey [64].

2.4. Anabolic steroids

Anabolic steroids are steroidal androgenic hormone, usually a steroid hormone, that obtained from either chemical synthesis or part of animals. Natural anabolic steroids are produced in porcine (pig), boar, horse, and sheep [75]. Among these, porcine is the most common source of anabolic steroids for wound healing especially the porcine placenta and testis. Anabolic steroids compose of four aromatic base structures which are three cyclohexane rings and one cyclopentane ring. There are more than 100 substances following molecular substitution to ring base. For porcine testis extracts, the highest contents of steroids are

19-nortestosterone (nandrolone) (MW = 274.4 g/mol), testosterone (MW = 288.43 g/mol), and 17β-estradiol (MW = 272.39 g/mol) [76]. They are easily soluble in alcohol but hardly soluble in water. Moreover, they have low stability that are easily oxidized [77–79]. Topical anabolic steroids have been used for wound treatment in the forms of solution and cream.

2.4.1. Processing methods

Chloroform with methanol extraction was used to extract crude materials [76,80]. Gamma irradiation and autoclaving were usually applied to sterilize porcine placenta extract [81].

2.4.2. Mechanisms on wound healing

The activity of anabolic steroids is to increase net protein preservation to the wound for new tissue formation process. They have an anticortisol activity that can decease catabolic response of cortisol, and decrease protein degradation without altering its anti-inflammatory response [82]. They also have direct effect on insulin-like growth factor-1 (IGF-1), TGF-β, and fibroblast growth factor (FGF) which play important roles in activation of cell proliferation, cell migration, collagen synthesis, tissue formation, and angiogenesis,[76,83,84]. leading to wound closure.

Wu C-H et al. found that proliferation of fibroblast cells cultured in porcine placental extracts (30 mg/ml) was significantly higher than that cultured in 0.5% fetal bovine serum (FBS) [85]. *In vi*vo test also showed that the TGF-β1 and bFGF levels in wound skin of rat which was treated with porcine placental extracts were significantly greater than that treated with normal saline at day 5–15 after burns. Healing time of the wound treated with porcine placental extracts was significantly less than that of normal saline group [85]. Lee D-M et al. have studied the topical anabolic steroid extracted from porcine testis for wound treatment [76]. Sixty rats with full thickness wound were divided into two groups which were cream + porcine testis-extracted steroid and cream without porcine testis-extracted steroid. The healing efficiency in terms of collagen formation and tensile strength of the wound treated porcine testis-extracted steroid was higher after 7 days of treatment.

2.4.3. Biological safety

The numbers of clinical study on topical anabolic steroids extracted from animals are very limit because the most of topical steroid are modified by chemical synthesis. One study has assessed the toxicity of cream + porcine testis-extracted steroid in wound healing by counting the while blood cells. There was no significant difference of WBC counts between cream + porcine testis-extracted steroid - treated group and cream without porcine testis-extracted steroid treated group. Skin rash was not found in both groups [76]. However, limitation of using the topical anabolic steroids is that the anabolic steroids could enter to blood circulation and cause the systemic effects [86].

2.5. Sericin

Sericin is a protein extracted from silk cocoons of *Bombyx mori*. It contains 18 amino acids. Serine, a moisturizing acid, is found around 30% of total amino acid content [87–89]. Following serine, aspartic acid and glycine are also found at around 10–20% [89,90]. The molecular weights of silk sericin depend on the extraction methods. Silk sericin obtained from heat extraction using autoclave at 120° C for 60 min has molecular weight around

25–150 kDa [90]. Silk sericin is a hydrophilic molecule which is water-soluble [91]. The secondary structure of silk sericin composes of two forms which are random coil and β-sheet. At high temperature (more than 50 °C), the major form of silk sericin is random coil which is easily soluble. On the other hand, at lower temperature, the major form of silk sericin is β-sheet which has strong hydrogen bonds, making it more difficult to dissolve and may form gel upon cooling [87]. Recently, various biological properties of silk sericin have been elucidated such as wound healing, antibacterial effect, and anti-inflammatory. It was then introduced to use in medical and pharmaceutical applications in various forms such as lotion, cream, ointment, gel, dressing, and bandage [87]. Sericin has been widely studied as accelerating agent for wound healing. Examples of clinical studies on topical wound treatment using silk sericin are shown in Table 4.

2.5.1. Processing methods

Silk sericin can be extracted by many methods including high temperature and high pressure degumming method using autoclave at 120 °C for 60 min, acid extraction using citric acid, alkaline extraction using sodium carbonate, and urea extraction [90]. Aramwit et al. found that sericin product from high temperature and high pressure showed the lowest toxicity to cells and showed the highest collagen promotion [90]. Gamma irradiation is reported to have high potential to sterilize the sericin scaffold and film [92,93].

2.5.2. Mechanisms on wound healing

Silk sericin is reported to accelerate proliferation of fibroblast cells and collagen synthesis [94]. Silk sericin from heat extraction using autoclave at 120 °C for 60 min activated the highest production of type I collagen and showed the lowest toxicity to cells at the concentration range of 8–100 μg/ml. Silk sericin from Chul Thai Silk 1/1 (0.2–1.0 mg/ml) can activate growth of L929 mouse fibroblast cells and the production of collagen type I in cell culture medium [95]. It could increase the number of skin fibroblast cells and collagen production to 250% of non sericin after 72 h [96]. Furthermore, it accelerated proliferation of mammalian cells including human epithelial cells and human hepatoblastoma [89]. For *in vivo* test, 3% silk sericin solution and 8% silk sericin cream significantly reduced wound size, increased healing score, and increased type III collagen formation without allergic reaction [97,98]. For burn wounds and split-thickness skin graft wounds, silk sericin could reduce wound healing time and pain score without sign of irritation [99–101]. Silk sericin could activate angiogenesis for wound healing process. Aramwit et al. found that the genipin-crosslinked sericin/PVA scaffolds could promote new blood vessel in full thickness wound together with vessel density increasing after 7 day of treatment [102]. Moreover, it can increase skin elasticity, and has anti-aging effect.

2.5.3. Antibacterial effects

Some researches found that silk sericin could inhibit bacterial growth. Fabric coating with silk sericin exacted by ethanol could inhibit *S. aureus* and *E. coli* in the disk diffusion test. Moreover, it decreased these bacteria more than 80% in a broth test [103]. Silk sericin extracted by alkali process (0.25% sodium bicarbonate) showed a potent antibacterial activity to *E. coli*. Silk sericin extracted by water degumming process possessed antibacterial activity to *S. aureus*. It caused membrane dysfunction and disturbed division and growth of both *E. coli* and *S. aureus* [104]. It also decreased colonies of gram positive bacteria when

Table 4. Clinical studies of topical wound treatment using silk sericin.

Study design	Wound type	Number of subjects	Control treatment	Outcome	Side effects
Randomize control trial [100]	Split-thickness skin graft donor site	30 (Split wound and treated one side with sample and another side with control)	Bactigras® (chlorhexidine paraffin gauze dressing)	• Healing time of the wound treated with 3% silk sericin was significantly less than that of the control group (12 ± 5 vs. 14 ± 5.2 days, respectively) • Pain score of the wound treated with 3% silk sericin was lower than that of the control group from day 1-4	No report
Randomize control trial [99]	Second degree burn wounds	29 (Sample 15, control 14)	Silver zinc sulfadiazine cream	• Healing time of the wound treated with silver zinc sulfadiazine cream containing 8% silk sericin was significantly less than that of the control group (22.42 ± 6.33 vs. 22.98 ± 9.27 days, respectively) • Percentage of wound size reduction of the wound treated with silver zinc sulfadiazine cream containing silk sericin -treated group was significantly higher than that of the control group after 14 and 42 days • Pain score of both groups were not significant difference • No infection was found in both groups	No severe adverse reaction in both groups
Randomize control trial [101]	Split-thickness skin graft donor site	30 (Split wound and treated one side with sample and another side with control)	Bactigras®	• Healing time of the wound treated with 1% silk sericin was significantly less than that of the control group (11 ± 6 vs. 14 ± 6 days, respectively) • Pain score of the wound treated with 1% silk sericin was significantly lower than that of the control group from day 1–5 • Transepidermal water loss index of the wound treated with 1% silk sericin was lower than that of the control group after healing days • No sign of infection was found in both groups	No systemic adverse reaction in both groups

the sericin concentration was increased [105]. However, some researches showed that the purified silk sericin extracted by degumming process without Tris-HCl and alkali did not have antibacterial effect [106].

2.5.4. Anti-inflammation effects

Silk sericin decreases the inflammatory reaction by suppressing cyclooxygenase 2 (COX-2) and nitric oxide genes in inflammation process. This effect was a concentration dependence [107]. The silk sericin nanoparticles also decreased the paw edema that induced by carrageenan injection. It decreased the number of cellular infiltration and inflammation reaction [108].

2.5.5. Moisturizing effect

The main amino acid of silk sericin is serine which is a moisturizing acid. Serine can preserve the skin moisture by generating a film on the surface of skin [88]. It was reported that 2% silk sericin gel can decrease transepidermal water loss and improve the smoothness of the skin [109].

2.5.6. Biological safety

Sericin was not toxic to the cell [95]. However, it was found that sericin at high concentration (more than 100 μg/ml) may decrease cell availability [90]. When sericin material was implanted subcutaneously in rats, the local effect in terms of the infiltration of inflammatory cells, necrosis, fibrosis and neovascularization showed that it was a non-irritating material [100,110].

2.6. Peptide

Peptides are biological molecules that are important factor in enzyme, healing process and other metabolic function of living organism. They are polymers that compose of amino acid residues linked with amide bond (carboxyl group and α-amino group linking) [111]. The molecular weight of peptides is less than 6000 Da, more than that is proteins [111]. Peptides have low stability and short half-life, so they are easily degraded. The biotechnology such as pH or temperature adjustment could control the stability of peptides [112]. Peptides can be obtained from chemical synthesis or extracted from natural sources especially amphibians. Amphibians such as salamander and frog have a special wound repair process. They can repair wound by regeneration like a mammalian fetal skin and show a perfect wound healing. In contrast, the wound repair process in a mammalian adult skin is the scar formation which lead to imperfect wound healing. Therefore, amphibian peptides were extracted and applied for wound treatment. The tylotoin extracted from salamander (*Tylototriton verrucosus*) is composed of 12 amino acid residues including 2 cysteines forming an intramolecular disulfide bridge [113]. AH90 was the peptides extracted from frog skin (*Odorrana grahami*). It composed of 24 amino acid residues [114].

2.6.1. Processing methods

Peptides from animal skin were extracted by freeze dried method [113,115]. Most of peptides that were extracted from amphibian skin were purified by gel filtration and HPLC [113,116]. Moreover, the peptides can be simply sterilized by filter-sterilization [117].

2.6.2. Wound healing activity

Peptides provide function in wound healing. They bound the cell surface integrin, leading to cell adhesion and migration. They also trigger the invasion of fibroblast or keratinocytes [118]. The transcription factor *Prx1*is one of the key factor that activates fibroblast in amphibians skin wound healing [119]. However, there are many peptides that involve in this process. The previous researches have investigated the peptides extracted from salamander and frog skin as wound healing accelerator. The results showed that topical treatment with tylotoin extracted from salamander at concentration 20 μg/ml accelerated full thickness wound healing process in mice. After 10 days, the wound areas of tylotoin-treated group were almost completely closed but the wound areas of the control group (vehicle) were closed around 74%. Moreover, tylotoin increased proliferation and migration of keratinocyte, fibroblast and vascular endothelial cells. It also enhanced secretion of TGF-β1 and IL-6 that are important factors in wound healing process [113]. Liu H et al. found that the AH90 extracted from frog skin at concentration 250 μg/ml significantly accelerated wound healing in mice compared with vehicle. It promoted re-epithelialization and TGF-β1 secretion to the wound. AH-90 also increased cell adhesion to fibronectin and laminin [114]. Frog skin was also applied in other pharmaceutical formulations including ointment. Mashreghi M et al. found that the frog skin extract in ointment significantly increased percentage of wound closure and increase area of neovascularization in rats comparing with ointment without the extract after 4 and 6 days' post-injury. Fibroblast cells and collagen production of the wound treated with frog skin extract were also significantly higher than those of the control group after 2, 4, and 6 days' post injury [115]. The results of Bazaz et al. demonstrated that frog skin powder in ointment significant accelerated wound contraction and enhanced amount of collagen after 6 days of treatment comparing ointment without powder. It significantly reduced inflammation after 2, 4, 6 days of treatment. Moreover, there was higher growth of new vessels in the frog skin extract powder in ointment treated group comparing with the powder treated group after 4 and 6 day of treatment [120].

2.6.3. Biological safety

The safety evaluation of peptides has not been reported Further researches on the sequence, stability and physical properties of these peptides were required

2.7. Proteoglycans

Proteoglycans (PGs) are complex extracellular macromolecules consisting of a core protein in which one or more covalently attached to glycosaminoglycans (GAGs) [121]. The chemical structure of PGs is complex. It composes of many types of proteins such as aggrecan, versican and GAGs including hyaluronan, chondroitin sulfate, and keratin sulfate [121]. The properties of PGs also depend on their chemical structure. For example, aggrecan with hyaluronic acid are structural components of ECM in cartilage, brain, intervertebral disc, tendon, and cornea [122,123]. They provide water retention, osmotic pressure, and proper collagen organization to the cell. Small leucine-rich proteoglycans are the most PGs that found in extra cellular matrix. They regulate cell process including migration, proliferation, and angiogenesis. Decorin that is a class I of small leucine-rich proteoglycans had an effect on collagen fibrillogenesis, fibroblast function, and wound healing. A part of cartilage, PGs structures are similar to EGF-like module which is the one factor in wound healing process [122,123]. Among various sources of PGs, the PGs extracted from salmon nasal cartilages

are widely studied as wound healing agent [124]. Aggregan with chondroitin sulfate are the major component of PGs extracted from salmon nasal cartilages [125]. Three glubular domains and two glycosaminoglycan-attachment domains are provided in its core protein. Its physical properties are a hydrated, and viscous gel because of the attached chondroitin sulfate chains with its domain [126]. Chondroitin 6-sulfate which has an effect on wound healing are also found in PGs extracted salmon nasal cartilage at around 60% comparing with bovine PGs (40%) [127]. Therefore, PGs from bovine are more studied as joint treatment and others than wound treatment [128,129].

2.7.1. Processing methods

The salmon cartilage was extracted by hydrochloric acid containing protease inhibitor or by acetic acid. It was freeze-dried and filtered by ultrafiltration method. Then, it was purified by anion exchanges chromatography and gel filtration exchange chromatography [125,127,130].

2.7.2. Mechanisms on wound healing

Proteoglycans bind growth factors, cytokines, enzyme and ECM components including FGF, IL-8, EGF, and collagen [131] and regulate cell proliferation, migration, and angiogenesis in tissue repair process and host defense mechanism [132,133].

In vitro test, PGs extracted salmon nasal cartilage at concentration 0.1–10 μg/ml could stimulate fibroblast proliferation and migration leading to wound healing acceleration. The important mechanism for wound closure was the interaction between chondroitin sulfate in the PGs and cell surface. This interaction activated intracellular signaling pathway to promote cell proliferation and migration. Moreover, PGs extracted from salmon nasal cartilage contained core polypeptide as EGF ligand to stimulate cellular proliferation and migration. However, this effect was reduced when the PGs concentration was 10–1000 μg/ml [124]. The cream containing 1 and 2% PGs extracted from fish cartilage was used to treat second degree burn wounds in rats. The results showed that the percentage of wound healing of PGs cream-treated group was higher than cream without PGs group after 7, 14, 21, and 28 days. Amount of PGs had an effect on wound healing. The cream containing higher PGs content (2%) promoted higher percentage of wound healing than that containing lower content (1%). Re-epithelialization and neovascularization of PGs cream-treated group were found to be greater than the control. The number of giant cells and macrophage were not significantly different and continuously decreased after 14 day [134].

2.7.3. Biological safety

There was no clinical report on the biological safety of using PGs for wound treatment. However, there was no abnormality in the rats treated with salmon cartilage powder containing PGs (1000 mg/kg/day) for 90 days. Moreover, there were no clinical side effects or abnormality in blood test in adults who received salmon cartilage powder containing PGs (1500 mg/kg/day) for 5 days [135].

2.8. Hyaluronic acid

Hyaluronic acid (HA) is an important component in synovial fluid and ECM. It contains disaccharide structure composed of D-glucuronic acid and N-acetylglucosamine [136]. The

polymer molecule of HA has molecular mass around 4000 kDa [137]. Hyaluronic acid can be synthesized by physiochemical process or extracted from bacteria and animals including rooster combs, bovine nasal cartilage, rabbit skin, shark skin, fish eyeball [137–139]. The highest amount of HA in extracted animals is rooster combs that have molecular mass around 2500 kDa. Therefore, the combs are the most popular source of HA production in medical materials [138]. Hyaluronic acid has effects on cell proliferation, differentiation, and tissue repair. Long chain HA (1000–5000 saccharides) activates fibroblast proliferation, migration and increases collagen deposit [140]. Small and medium chain HA (4–25 and 1000–1250 saccharides) involve in angiogenesis, and inflammation [136,140]. Hyaluronic acid molecule can dissolve in water to form viscous gel and contains hygroscopic property [141]. However, it has the problem about low stability. The esterification method is used for modified HA structure to improve stability and reduce degradation [140].

2.8.1. Processing methods

Hyaluronic acid was extracted with acetone, distilled water, and sodium chloride solution [142]. Purification process of HA are protease digestion, HA ion-pair precipitation, protein electrodeposition, ultrafiltration, HA non-solvent precipitation, and lyophilization [138,139].

2.8.2. Mechanisms on wound healing

In wound healing process, HA can bind to fibrinogen influenced clotting pathway. At the high concentration of HA, a porous network structure is formed to diffuse cells and proteins for cell migration [136]. For angiogenesis, HA binds to CD44 supporting new blood vessels formation [143]. It can activate keratinocyte proliferation, migration and increase collagen deposit leading to wound healing [141]. HA reduced pain by decrease of nerve sensitivity [137]. Moreover, it can control the hydration environment between wound healing process because of its hygroscopic property [144]. Cerqueira et al. studied in full thickness wounds of rats. They demonstrated that the percentage of wound closure treated with gellan gum HA spongy-like hydrogel was significant higher than the wound without treatment. Vessel density in gellan gums HA spongy-like hydrogel treated group was also significant higher than without treatment group after 7 days' post injury [145]. Humbert et al. studied in 89 leg ulcer patients. They found that percentage of wound size reduction and numbers of wound healing of 0.05%HA gauze pad treated group was significant higher than gauze without HA. Moreover, pain score of 0.05%HA gauze pad treated group was also significant less than gauze without HA [146]. Wound healing time of secondary burn wound treated with 0.2% HA and 1% silver sulfadiazine was also significantly less than wound treated with only 1% silver sulfadiazine [147].

2.8.2. Anti-inflammation effects

Exogenous HA could reduce the production and activity of inflammatory mediators and MMPs [137]. It can interact with TNF-stimulated gene-6 (TSG-6) to decrease neutrophil migration leading to inflammatory reduction [136].

2.8.3. Biological safety

HA from rooster combs showed better safety profile than HA that extracted from Streptococci bacteria [148]. No adverse event was observed in the HA cream treated group [147]. However, it may induce allergic reaction especially in avian allergy history [138].

2.9. Keratin

Keratin is a protein that contains high amount of cysteine. It is found in human skin and hair. The structures of keratin are polypeptide chain together with fine filament-matrix structure that have been divided into two form; alpha and beta structure. In animals, the alpha keratin is found in wools, hair, skin, nails, hooves, and horns of mammal structure. The beta keratin is found in feathers, avian beaks and claws, reptilian claw and scales. Both of keratin structures are found in reptilian epidermis and pangolin [149]. The molecular mass of alpha keratin contains is around 40–68 kDa and beta keratin is around 10–22 kDa [149]. Keratin is insoluble material so modified chemical reaction including acid, base, enzyme, and sulfitolysis method are used for medical preparation [150]. For wound healing, keratin was produced in many form including powder, gel, dressing, scaffolds.

2.9.1. Processing methods

Keratin was extracted by acid, alkali, high concentration of salt solution [151], oxidation [152], reduction [153], ion liquid extraction [154], or sulfitolysis [155,156]. Soxhlet apparatus or dialysis was used for removal external lipid and impurities [155–157].

2.9.2. Mechanisms on wound healing

The mechanisms of action of exogenous keratin to promote wound healing are to stimulate keratinocyte, induce endogenous keratin expression, and accelerate epithelialization together with upregulation migration and proliferation of keratinocytes [158]. It activates keratinocytes through expression of KRT6, KRT16, and KRT17 genes [159]. *In vivo* test, Park et al. reported that the full thickness wound size of keratin hydrogel treated group was significantly lower than hydrocolloid dressing treated group and non-treated group at day 7 and 14 post' injury. Amounts of fibroblast and epithelial cells of keratin hydrogel treated group were also significantly higher than hydrocolloid dressing treated group and non-treated group at day 7 post' injury. Collagen deposition in keratin treated group was also higher than control [155]. The keratin hydrogel also showed the same results in Wang et al. study. It significantly accelerated wound healing comparing with non-treated group in rats. Cell proliferation and neovascularization were found in keratin hydrogel treated group in 7 days. After 14, and 21 days, collagen deposition in hydrogel treated group was higher than non-treated group [160]. In clinical research, Davidson et al. demonstrated that epithelialization rate of keratin dressing treated group was significantly higher than alginate dressing treated group in the elderly patients with partial thickness wound [158]. Keratin gel also significantly decreased wound healing time in superficial and partial thickness burn patients comparing with standard care (non-adherent dressings and topical liquids) [161]. Moreover, keratin could induce coagulation mechanism including platelet adhesion, fibrin polymerization [153,162,163]. *In vitro* test, Wang et al. found that extracted feather keratin from chicken significantly reduced whole blood clotting time comparing without keratin [153]. In rat tail amputation and liver scratch experiments, blood clotting time and mass of blood loss of keratin treated group were significantly less than non-keratin treated group [153].

2.9.3. Biological safety

Keratin was biocompatibility and safety for wound healing. No adverse effect was observed in keratin treated group [158]. Immunotoxicity and systemic toxicity was also not found in keratin treated group [160].

Table 5. Advantage and disadvantage of animal-derived products in topical wound application.

Animal-derived products	Advantage				Disadvantage
	Wound healing promotion	Anti-bacteria	Anti-inflammation	Safety	
Chitosan	+	+		+	• Molecular weight has significant effect on wound healing properties • Allergy in patients who are allergic to arthropods including shrimp, crab, insects, and shell fish
Collagen	±			+	• Fast degradation rate • Concerning zoonosis contamination • Case report of allergic reaction to bovine collagen • Expensive
Honey	+	+	+	+	• Antibacterial property can be decreased when in contact to wound exudate except manuka honey • Pain in sensitive nerve ending patients • local atopic reaction
Anabolic steroids	+			+	• Absorbed to blood circulation, leading to systemic side effect
Sericin	+	±	+	+	• Cell viability is decreased at high concentration • Antimicrobial activity depends on degumming process
Peptide	+			+	• Limited research document
Proteoglycan	+			+	• Poor wound healing property at high concentration • Limited research document
Hyaluronic acid	+		+	+	• May induce allergic reaction especially in avian allergy history
Keratin	+			+	• Chemical modified process of keratin extraction may not be friendly with environments

Notes: +: positive activity; ±: may have positive or negative activity.

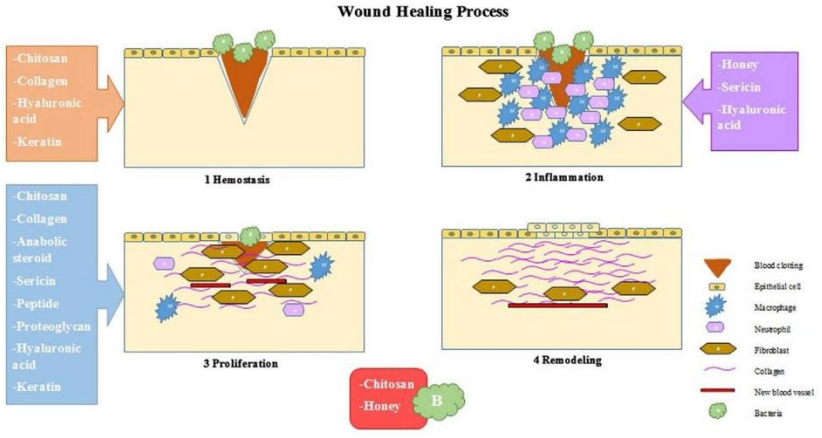

Figure 1. Wound healing mechanisms of animals products and extracts.

The advantages (in terms of wound healing promotion, anti-bacteria, anti-inflammation, and safety) and disadvantages of these animal-derived products and extracts are demonstrated in Table 5. Moreover, wound healing mechanisms of these animal-derived products and extracts were concluded in Figure 1.

3. Conclusion

The agents from animals including chitosan, collagen, honey, anabolic steroids, silk sericin, peptides, and proteoglycan can promote wound healing. Moreover, they may contain other activities such as anti-inflammatory, antimicrobial activity, and moisturizing effect which can also improve wound healing property. In addition, the biocompatibility and safety of these agents are needed to be considered. The disadvantage such allergy, low stability, high cost may be found. Some animal-derived products required more researches and documents to support its wound healing properties.

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Disclosure statement

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Development of bacterial cellulose incorporating silk sericin, polyhexamethylene biguanide, and glycerin with enhanced physical properties and antibacterial activities for wound dressing application

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ABSTRACT

Bacterial cellulose (BC) has been applied for wound dressing application. In this study, physical and biological properties of the BC dressing were improved by incorporation of silk sericin (SS), polyhexamethylene biguanide (PHMB), and glycerin. The glycerin incorporation reduced dehydration rate and wound adhesion of the BC dressing in a concentration-dependent manner. PHMB, an antiseptic agent, provided antibacterial activity against Gram-positive and Gram-negative bacteria. Meanwhile, SS would enhance collagen and tissue formation in wounds. Finally, we confirmed that the BC dressing incorporating SS, PHMB, and glycerin was safe to be used as a medical material according to ISO 10993-6 standard.

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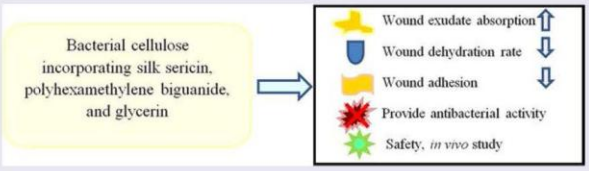
GRAPHICAL ABSTRACT



1. Introduction

Wound healing is a complicated process involving multiple factors that are crucial for the repair of the injured area [1]. The healing time depends on nutrition status, age, systemic diseases, and the wound environment, especially bacterial infection. Thus, one aim of promoting the healing process is to control the wound environment appropriately [2]. Accordingly, the basic strategy to control the wound environment is treatment with a wound dressing. An ideal wound dressing should be able to maintain a moist environment to the wound, which is necessary for pain reduction and tissue-forming promotion [3]. Moreover, it should activate collagen synthesis, allow oxygen permeation, and absorb exudate from wound. To reduce pain upon removal, the wound dressing should not be adherent to the wound surface. It may also possess an antibacterial effect to prevent infection of the wound. Last, it should be cost effective [4–6].

Bacterial cellulose (BC) is a kind of cellulose that is synthesized by nonpathogenic bacteria, such as *Acetobacter xylinum*, in an appropriate static media [7–9]. The naturally derived BC is biocompatible and minimally toxic [10]. Another advantage of BC is that it can be produced from several kinds of agricultural products that are cheap and can be scaled up to a commercial level easily. Due to its ultrafine network structure, BC has high water absorption capacity and oxygen permeation [11]. BC dressings are, therefore, reported to provide wound exudate absorption ability, a controlled moist environment, and a cooling effect for pain reduction in wound dressing application [12–14]. In previous studies, it was found that the BC dressing was not only effective for healing of burn and chronic ulcers [12,15] but also as a potential topical drug-delivery system [11]. Drug or active compounds could be loaded and released in a controlled manner from the BC dressing [16].

Silk sericin (SS) is a water-soluble protein extracted from the silk cocoon (*Bombyx mori*) [17]. SS is reported to activate proliferation of fibroblast cells and type I collagen synthesis without causing inflammation or toxicity [18–21]. It is widely used as an active compound in several applications, including cosmetic and medical products. Previously, we

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found that SS cream and wound dressings that released SS decreased the wound healing time in rats [22] and in human split-thickness skin graft donor sites [23]. Therefore, SS is considered as a tissue-forming promoter for wound healing process.

In terms of antibacterial activity, polyhexamethylene biguanide (PHMB) is an antiseptic agent that has potential against both Gram-positive and Gram-negative bacteria and with low toxicity toward human cells [24,25]. PHMB binds phospholipids of the bacterial membrane and disturbs the lipid bilayers, thus causing ion leakage and loss of function, which consequently results in cell death [26,27]. Furthermore, it shows low skin irritation [28,29] and has a higher efficacy than povidone iodine and chlorhexidine after long-term exposure [30]. It has been reported that PHMB can reduce bacterial infection in acute and chronic wounds, leading to an accelerated healing rate [31,32] without development of micro-organism resistance [33,34].

In addition to a tissue-forming promoter and antibacterial agent, a good humectant, such as glycerin, adds some advantageous properties to the dressing. Glycerin—a glycerol in water—is a clear, odorless, colorless, and viscous liquid, and it is highly soluble in water and alcohols [35]. It has been used as a water-absorption ingredient in cosmetics, food, and pharmaceutical products [36]. For wound dressings, glycerin has an advantage in terms of protecting against water loss protecting, changing plasticity [37], and increasing hydration [38,39] with minimal toxicity [40]. It also reduces the adhesive property of the wound dressing [41,42].

In this study, to obtain a wound dressing with improved properties, a BC dressing was combined with SS, PHMB, and glycerin. The advantageous effects of these components were expected to be found in this BC dressing. Furthermore, it was hypothesized that the glycerin incorporation would alter some important characteristics of the BC dressing. Herein, the amount of glycerin incorporated into the BC wound dressing was optimized. The physical and biological properties and safety of this dressing were investigated. Physical properties in terms of absorption of wound exudate, dehydration rate, and adhesive property of the wound dressing were assessed. Antibacterial activity of the wound dressing against Gram-positive and Gram-negative bacteria was assessed. Last, the developed BC dressing incorporating SS, PHMB, and glycerin was subcutaneously implanted into Wistar rats to evaluate its safety and was compared to the clinically available products, Suprasorb X and Bactigras.

2. Experimental

2.1. Materials

Bacterial cellulose (BC) was produced from the *A. xylinum* strain (Kasetsart University, Bangkok, Thailand) in coconut water medium following the method of Verschuren et al. [43] with a slight modification. Briefly, 50 g sucrose, 5 g ammonium phosphate [$(NH_4)_3PO_4$], and 10 mL acetic acid (CH_3COOH) were added into 1 L of boiled coconut water. The solution was adjusted with CH_3COOH to pH 4.5. Then, 10 mL of *A. xylinum* was added to the adjusted solution and transferred to fermentation molds. The molds were maintained at 30°C under sterilized static conditions for 10–12 days to form bacterial cellulose. After that, the BC was washed with 2% w/v aqueous sodium hydroxide (NaOH) solution at 70°C until neutral pH is reached. Glycerin was of analytical grade and was from Ajax Finechem, Australia. Acetic acid, sodium hydroxide, ammonium phosphate, and other chemicals were of analytical grade and purchased from Sigma-Aldrich (USA). Silk sericin solution was prepared from *B. mori* cocoons supplied by Chul Thai Silk Co., Ltd (Petchabun Province, Thailand) using a high-temperature and high-pressure degumming method [19]. PHMB was kindly provided by Lonza Group Ltd (Basel, Switzerland). Bactigras (0.5% chlorhexidine acetate in a white soft paraffin) was purchased from Smith & Nephew Co., Ltd (London, UK). Suprasorb X + PHMB (0.3% PHMB in bacterial cellulose wound dressing) was purchased from Lohmann & Rauscher GmbH & Co. KG (Neuwied, Germany).

2.2. Preparation of the BC dressing incorporating SS, PHMB, and glycerin

Bacterial cellulose (10 × 10 × 0.01 cm) was subsequently loaded with three agents as follows. First, 1% w/v SS was dropped on BC dressing (2 mL per side) and incubated at 4°C for 2 h. Second, 0.3% w/v PHMB was dropped on the dressing (5 mL per side) and incubated at 4°C for another 2 h. Last, glycerin at different concentrations (0, 10, 20, 30, 40, 50, and 60%) was absorbed on dressing. Between each step of loading, the dressing was left to dry before loading the next step to ensure the complete adsorption of previous agent. In other words, the dressing adsorbed the whole amount of loaded agents for all three steps. The SS/PHMB/glycerin bacterial cellulose dressings were obtained.

2.3. Methods

2.3.1. Exudate absorption test using a wound bed model

To mimic the absorption of exudate from wounds, a blue agar plate test was used as a model [44]. The sample dressings (2 × 2 × 0.1 cm) were placed on a blue agar plate (0.6% w/v agar in 0.9% w/v saline solution with 0.1% v/v bromophenol blue dye) and incubated at 37°C for 7 days. The blue agar plate composed of two parts, which are agar, representing wound tissue, and blue solution, representing wound exudate. The color of the blue agar plates after the removal of dressings is evaluated using a chroma meter (Konica Minolta Model: CR-400, Japan) and is reported as the b value (a negative b value represents a change in the color to blue) at the seventh day ($n = 3$).

2.3.2. Dehydration test

The sample dressings (2 × 2 × 0.1 cm) were immersed in 1 mL of reverse osmosis water. After complete absorption (maximum loading), the dressings were incubated at 37°C. At different periods, the dressings were weighed and their

dehydration percentages were calculated using the following Eq. (1):

$$\text{percentage dehydration } (\%) = (W_0 - W_t)/W_0 \times 100 \quad (1)$$

where W_0 and W_t represent the initial weight and the weight of the dressing at each time point, respectively ($n = 3$).

2.3.3. Peel test

Porcine skin obtained within 2 h of sacrifice was used for the experiment. The wound was created on porcine skin at a depth of 1 cm (representative of a full-thickness skin wound), length of 20 cm, and width of 3 cm. The sample dressings (2.5 × 15 × 0.1 cm) and Bactigras (control) were randomly attached on the wound ($n = 3$). After 12 h of attachment, the adhesive force applied to peel the dressings off was evaluated using a universal testing machine (Instron, no. 5567) with 1 kN constant loading, tensile rate of 5 mm/min, and a fixed peeling angle of 135°. The adhesive force was calculated at the steady state of peeling force ($n = 3$) [23].

Furthermore, the number of cells attached to the dressings after being peeled off from the wound was analyzed by fluorometric quantification of cellular DNA [45]. Hoechst dye solution was used to stain the DNA of cells, and the fluorescent intensity was evaluated at 355 nm (excitation) and 460 nm (emission) using a fluorescent microplate reader ($n = 3$).

2.3.4. Antibacterial test

The antibacterial effect of the dressings was tested using disk diffusion method (CLSI M2-A9). Six bacteria were chosen for the test: *Bacillus subtilis* (ATCC 6633), *Staphylococcus aureus* (ATCC 25923), and methicillin-resistant *S. aureus* (MRSA) of Gram-positive bacteria, and *Escherichia coli* (ATCC 25922), *Acinetobacter baumannii* (ATCC 19606), and *Pseudomonas aeruginosa* (ATCC 27853) of Gram-negative bacteria. All bacterial strains were cultured on a Müller–Hinton (MH) agar plate at 37°C for 24 h to prepare bacterial solutions. Three to five colonies of each bacteria were selected and transferred to 5 mL of tryptone soy broth and incubated at 37°C for 4–6 h until the final concentration of bacteria was 1.5×10^8 CFU/mL, as measured by a UV/VIS spectrometer (Lambda 25, PerkinElmer, Waltham, MA, USA) at 625 nm. The bacterial solution was swabbed on the MH agar plate. Then, the sample dressings and the Bactigras (control) (1 × 1 cm) were placed onto the plate and incubated at 37°C. After 24 h, the inhibition zone was evaluated.

2.3.5. In vivo *safety test*

The *in vivo* safety test of samples was performed following the ISO 10993-6 standard: biological evaluation of medical devices (part 6: tests for local effects after implantation). The sample dressings, Suprasorb X + PHMB, and Bactigras (1 × 1 cm) were subcutaneously implanted into 16 healthy female Wistar rats (8 weeks old, 200–300 g) those were received from National Laboratory Animal Center, Mahidol University. One rat received three samples randomly. Suprasorb X + PHMB and Bactigras were used as control groups because they are clinically used dressing. The animal experiments were performed according to the Chulalongkorn University Animal Care and Use Committee (CUACUC 13/57) under standard sterile conditions. The test was approved by the Ethics Committee of the Faculty of Medicine, Chulalongkorn University. Briefly, subcutaneous tissue was incised (1.5 × 1.5 cm), and the dressing sample was inserted into each pocket by block randomization (block size 3). Then, the wound was sutured and disinfected with povidone–iodine solution. The rats were kept in stainless steel cage (1 rat/cage) at strictly hygienic conventional room and were fed with standard diet. After 7, 14, 21, and 28 days of implantation, four rats were euthanatized using carbon dioxide inhalation. The samples and surrounding tissues were collected and fixed in 10% v/v formalin solution. Subsequently, the embedded samples were sectioned and stained with hematoxylin and eosin (H&E). For histological assessment, the inflammatory cell types, neovascularization, fibrosis, and fatty infiltrate were semiquantitatively scored following ISO 10993-6 standard and were assessed by pathologist (double-blind) at two different times. The score composed of four levels (0 = not observed, 1 = rare, 2 = minimal, 3 = heavily infiltrated, and 4 = packed infiltrate), and the final score was calculated following Eq. (2):

$$\text{final score} = (2C_t + M_t) - (2C_c + M_c) \quad (2)$$

where C is the total score of polymorphonuclear cells, lymphocytes, plasma cells, macrophages, giant cells, and necrosis of the sample, M is the total score of fibrosis, neovascularization, and fatty infiltrate of sample (t = test sample and c = control). The final score was classified as follows: 0.0–2.9 (non-irritant), 3.0–8.9 (slight irritant), 9.0–15 (moderate irritant), and >15 (severely irritant). The level of irritation was compared with the control [46].

2.4. Statistical analysis

SPSS version 17.0 (SPSS Co., Ltd, Bangkok, Thailand) was used for statistical evaluation. One-way ANOVA was calculated with statistical significance at $p < 0.05$ for comparing test groups with the control, and a *post hoc* test evaluated the difference between groups at different times.

3. Results

3.1. Absorption ability of the dressings

Figure 1a presents the images of the BC dressings after adsorption with the blue agar wound exudate model. A blue color could be seen on all SS/PHMB/BC dressings and BC dressings. The negative b values, which indicate the blue color on the blue agar gel after the removal of dressings, are reported in Figure 1b. Low negative b means blue agar was adsorbed a large amount of exudate. Each agar plate beneath the BC dressing and the SS/PHMB/BC dressings showed low negative b values compared to the blue agar gel, which indicates that all SS/PHMB/BC dressings and the BC dressing could adsorb wound exudate and had a similar absorption ability. Addition

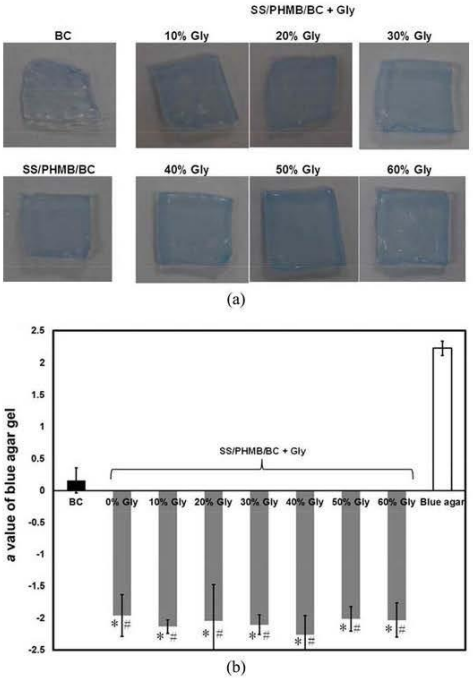

Figure 1. (a) Macroscopic images of the BC dressing and SS/PHMB/BC dressings with 0–60% glycerin after adsorbed with blue agar wound exudate. (b) *b* value of blue agar gel after the dressings was removed, determined by a chromameter (minus *b* value represents a change in the color to blue). * and # significant difference when compared with the blue agar gel and BC dressing, respectively ($p < 0.05$). *Note*: PHMB, polyhexamethylene biguanide.

of SS, PHMB, and glycerin into the BC dressing did not decrease its absorption ability.

3.2. Dehydration ability of the dressings

The percentage weight losses of dehydrated dressings are shown in Figure 2. For all dressings, a high weight-loss percentage of the dressings was observed initially and then it became steady after that. The weight-loss percentages of the SS/PHMB/BC dressings with 10–60% glycerin were lower than that of the BC dressing. The weight loss of the dressings was inversely proportional to the percentage of glycerin incorporated, and the SS/PHMB/BC dressing composed of a highest amount of glycerin (60%) and had a lowest percentage weight loss during the assay.

3.3. Adhesive property of the dressings

The adhesive properties of the dressings are presented in terms of adhesive force applied to peel off the dressings (Figure 3a) and the number of cells attached to the dressings after being

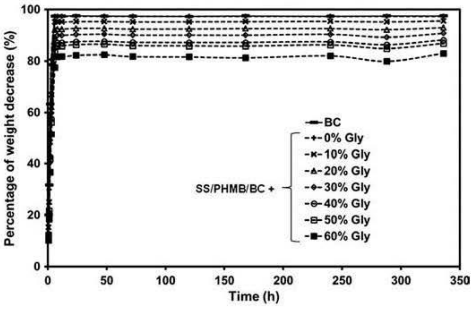

Figure 2. The percentage of weight loss of BC dressing and SS/PHMB/BC dressings with 0–60% glycerin after incubated at 37°C for different periods. *Note*: PHMB, polyhexamethylene biguanide.

peeled off (Figure 3b). The adhesive forces applied to peel off all BC dressings were significantly less than that of the Bactigras. The incorporation of glycerin slightly decreased

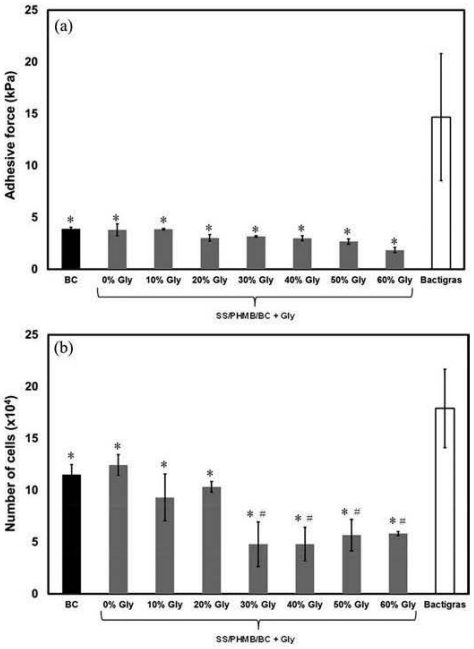

Figure 3. (a) Adhesive force applied to peel off the BC dressing, SS/PHMB/BC dressings with 0–60% glycerin, and Bactigras from wound on porcine skin. (b) Number of cells attached on the BC dressing, SS/PHMB/BC dressings with 0–60% glycerin, and Bactigras after peeled off from wound on porcine skin. * Significant difference when compared with the Bactigras ($p < 0.05$), # significant difference when compared with the BC dressing ($p < 0.05$). *Note*: PHMB, polyhexamethylene biguanide.

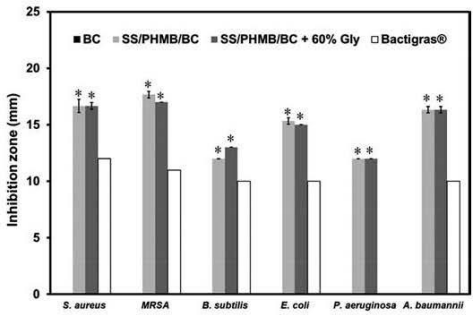

Figure 4. Inhibition zone of the BC dressing, SS/PHMB/BC dressings without and with 60% glycerin, and Bactigras on *B. subtilis*, *S. aureus*, MRSA, *E. coli*, *A. baumannii*, and *P. aeruginosa* agar plate. * Significant difference when compared with the Bactigras ($p < 0.05$). *Note*: PHMB, polyhexamethylene biguanide; MRSA, methicillin-resistant *S. aureus*.

the adhesion of the BC dressings. Furthermore, significantly fewer cells were found on the BC dressings compared to the Bactigras. A high amount of glycerin incorporated into the BC dressings (30–60%) resulted in a significantly lower number of cells being attached.

3.4. Antibacterial activity of the dressings

Figure 4 shows the bacterial inhibition zones of the dressings after being incubated with Gram-negative and Gram-positive bacteria for 24 h. The BC dressing without incorporation of SS, PHMB, and glycerin did not inhibit the growth of any strains of bacteria. On the other hand, the BC dressings with SS and PHMB incorporated strongly inhibited the growth of all bacteria. The bacterial inhibition zones of SS/PHMB/BC dressings without and with 60% glycerin were significantly larger than those of Bactigras for all bacterial strains. The presence of 60% glycerin did not affect the bacterial inhibition of the BC dressing.

3.5. In vivo safety of the dressings

The standard safety test was performed according to ISO 10993-6 to evaluate the safety of the SS/PHMB/BC dressing with 60% glycerin in comparison with the clinically available Suprasorb X + PHMB and Bactigras. All rats implanted with dressings were healthy and had no signs of inflammation (pain, swelling, redness, heat). Figure 5 shows the images of H&E-stained sections of the implanted dressings. Excessive infiltration of inflammatory cells or other signs of infection were not observed with any dressing. The intensity of inflammatory cells, necrosis, fibrosis, neovascularization, and fatty infiltrate was graded as presented in Table 1. Overall, the intensity of all signs of infection in the SS/PHMB/BC dressing with 60% glycerin was comparable to that of Suprasorb X + PHMB and Bactigras. The implantation of the SS/PHMB/BC dressing with 60% glycerin did not irritate the surrounding tissue throughout the implantation period.

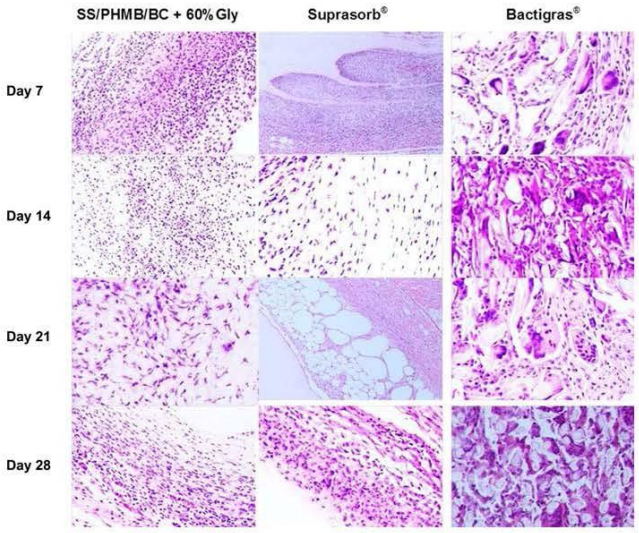

Figure 5. Microscopic images of H&E-stained sections indicating inflammatory cells of SS/PHMB/BC dressings with 60% glycerin, Suprasorb X + PHMB, and Bactigras at 7, 14, 21, and 28 days (scale bar = 30 μm). *Note*: PHMB, polyhexamethylene biguanide.

Table 1. Average intensity of inflammatory cells, necrosis, fibrosis, neovascularization, and fatty in the SS/PHMB/BC + 60% Gly dressing, Suprasorb, and Bactigras after subcutaneous implantation for 7, 14, 21, and 28 days.

	7			14			21			28		
Day	A	B	C	A	B	C	A	B	C	A	B	C
Polymorphonuclear leukocytes (PMN)	4[a]	4	3	4	3	0	3	2.3	0	3.8	2.8	0
Lymphocytes	3	1	3	2	2	2.3	1.5	2	2	2	1.8	2
Plasma cells	0	0	0	0	0	0	0	0	0	0	0	0
Macrophages	1	2.5	3	3	1	2.5	1	2.3	1.5	2	1.5	2
Giant cells	0	0	3	0	0	4	0	0	4	0	0	4
Necrosis	0	0.5	2	0	0.5	0	0	0.5	0	0	0	0
Fibrosis	2	2	3	2	2	3.8	1.3	1	1.5	2	2	3
Neovascularization	3	2.5	4	2	2.3	2.3	1	2.5	1.5	2	2	3
Fatty infiltrate	2.5	2.8	2	1	0	4	0	1.8	2.5	0	0	3.5
Total [calculated formula (2)]	23.5	23.3	37	23	17.3	27.5	13.3	19.3	20.5	19.5	16	25.5
Score (test −control)	−13.5	−13.7		−4.5	−10.2		−7.2	−1.2		−6	−9.5	
Ranking of irritant	Non	Non		Non	Non		Non	Non		Non	Non	

A, SS/PHMB/BC + 60% Gly dressing; B, Suprasorb; C, Bactigras.
[a]Intensity 0–4: 0 = not observed, 1 = rare, 2 = minimal, 3 = heavily infiltrate, and 4 = packed infiltrate.
Final score = 0.0–2.9 (non-irritant), 3.0–8.9 (slight irritant), 9.0–15 (moderate irritant), and >15 (severely irritant).

4. Discussion

In our previous work, an SS-releasing BC gel was developed to be applied as a bioactive mask for facial treatment [47]. The gel showed appropriate physical and biological properties, while silk sericin could be control-released from the gel to continuously promote the collagen production of skin cells [48]. In the present study, this SS-releasing BC gel was used as a wound dressing. It has been widely reported that the BC gel has high water absorption capacity, oxygen permeation, and wound exudate absorption ability and maintains a moist environment [12–14], which are all the essential properties of an ideal wound dressing.

However, the BC dressing still lacks the tissue-forming promotion and antibacterial activities. It was shown previously that SS could activate collagen production, epithelialization, and tissue formation in wounds [17,19,22]. Thus, SS was incorporated into the BC dressing for this purpose. The controlled release of SS from the BC gel and the continuously promoted proliferation of fibroblasts, collagen production, and epithelialization in wounds were demonstrated previously [23,47,49,50]. To introduce a direct antibacterial effect against various bacteria, PHMB was also incorporated into SS-releasing BC dressings. PHMB has been reported to show effective antibacterial activity against both Gram-negative and Gram-positive bacteria [24,25]. Herein, it was shown that the SS/PHMB/BC dressing without or with 60% glycerin could inhibit the growth of *B. subtilis*, *S. aureus*, MRSA, *E. coli*, *A. baumannii*, and *P. aeruginosa*, comparable to the Bactigras, which is the commercial wound dressing composed of chlorhexidine as an antiseptic (Figure 4).

Additionally, glycerin incorporated in the BC dressing was supposed to reduce the adhesion of the wound dressing over wound surface due to its lubricant property, consequently resulted in less pain. We here showed that the incorporation of glycerin, particularly at 30–60%, produced a less-adhesive BC dressing, as confirmed by the lower adhesive force and the fewer number of cells attached (Figure 3). Stout and McKessor [42] reported that glycerin-based hydrogel was a nonadhesive dressing and did not cause surrounding wound damage.

Furthermore, glycerin incorporation reduced the dehydration rate of the BC dressing in a concentration-dependent manner (Figure 2). This can be explained by the humectant and hygroscopic nature of glycerin, which attracts and holds water molecules from the surrounding environment. It was reported that glycerin could swell the outer layer of the stratum corneum to keep the skin moist and to prolong the shelf life of the product [35,51]. We also proved that the incorporation of glycerin retained the wound exudate absorption ability (Figure 1) of the BC dressing. Therefore, our developed BC dressing would be appropriate for moderate to high exudate wounds.

Last, the safety of the SS/PHMB/BC dressings with 60% glycerin according to ISO 10993-6 standard is shown (Table 1 and Figure 5). No signs of inflammation or infection were observed on our developed BC dressing because all components at this appropriate concentration were reported to be safe for human skin [19,24,40,52].

5. Conclusion

The physical properties and activities of BC were improved by incorporation with SS, PHMB, and glycerin. SS/PHMB/Gly bacterial cellulose dressing provided antibacterial activity. Glycerin incorporation reduced the adhesive property and dehydration rate of the BC dressing in a concentration-dependent manner. Furthermore, it was found that the resultant SS/PHMB/BC dressing with 60% glycerin could absorb a high amount of wound exudate and was safe to be used as a wound dressing or as a medical material, comparable to the clinically available dressing, Bactigras. The clinical efficacy and safety of this dressing will be further evaluated in future work.

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Identification and quantification and antioxidant activity of flavonoids in different strains of silk cocoon, *Bombyx mori*

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ABSTRACT

Silk cocoon is produced from silkworm (*Bombyx mori*) to protect itself from outer environment. Various strains of cocoon contain different forms and amounts of flavonoids, which may affect on their antioxidant activity. Moreover, the extraction method would influence the amount of flavonoids extracted. Therefore, the objectives of this study were to identify and quantify the flavonoids in 3 strains of bivoltine *Bombyx mori* silk cocoon (Chul 1/1; white cocoon, Chul 3/2; greenish cocoon, and Chul 4/2; yellow cocoon) extracted by 6 different solvents including acetone, ethyl acetate, dimethyl sulfoxide (DMSO), ethanol, methanol, and purified water. The flavonoids extracted were identified and quantified by liquid chromatography–mass spectrometry (LC-MS). The antioxidant activity of flavonoids extracted was also investigated by visible spectroscopy at 517 nm. The results showed that Chul 3/2 silk cocoon contained the highest amount of flavonoids. Purified water seemed to be the best solvent that preserved most antioxidant activity of the flavonoids extracted. Flavonoids in Chul 1/1 and Chul 4/2 silk cocoon were rarely found, however they contained some antioxidant activities. The data from this study can provide basic information for flavonoid extraction from silk cocoon which can also apply for other flavonoid-containing natural biomaterials.

1. Introduction

Silk cocoon is produced from silkworm (*Bombyx mori*) to protect itself from outer environment. It composes of many advantageous substances including proteins and pigments. The proteins, sericin and fibroin, are widely studied for medical applications due to their good physiochemical properties and biological activities such as anti-oxidation, anti-inflammatory, biocompatibility, acceleration of cell proliferation, and activation of collagen synthesis [1]. On the other hand, pigments in silk cocoon also have antioxidant activity. The compounds in these pigments depend on food of larvae (mulberry leaves) and silk worm strain [2,3]. The phenolic compound including flavonoids from mulberry are transferred and modified within silkworm by glucosyltransferase to accumulate in silk cocoon, [4–6]. Flavonoids in silk cocoon were found in various forms such as quercetin or kaempferol derivative [3]. It was demonstrated that different strains of cocoon contains different forms and amounts of flavonoids, which may affect their antioxidant activities. Moreover, the extraction method would influence the amount of flavonoids extracted. Prommuak et al. reported that the highest amount of flavonoids were found in ethanol extraction at 70 °C for 12 h but the yield decreased at higher temperature [7]. Nonetheless, Yamzaki et al. found that 80% methanol could extract the high amount of flavonoids from yellow-green silk cocoon [8]. However, the comparative study on the extraction methods for identification and quantification of flavonoids in different strains of silk cocoon has not been well document. Therefore, the objectives of this study were to identify and quantify the flavonoids extracted from 3 strains of silk cocoon using various extraction solvents including acetone, ethyl acetate, dimethyl sulfoxide (DMSO), ethanol, methanol, and purified water. Moreover, the antioxidant activity of the flavonoids extracted was also investigated.

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2. Materials and methods

2.1. Materials

Silk cocoon from *Bombyx mori* silkworm of 3 different strains (Chul 1/1; white cocoon, Chul 3/2; greenish cocoon, and Chul 4/2; yellow cocoon) were supplied from Chul Thai Silk Co., Ltd. (Petchaboon, Thailand). Quercetin and kaempferol standards were purchased from Extrasynthèse (Genay, France). Acetone, ethyl acetate, dimethyl sulfoxide (DMSO), ethanol, methanol, sulfuric acid, hydrochloric acid, DPPH (2,2-diphenyl-1-picrylhydrazyl), trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), rutin standard, and other chemicals were purchased from Sigma-Aldrich Handels GmbH (Vienna, Austria).

2.2. Methods

2.2.1. Identification of flavonoids in silk cocoons

Silk cocoons were cut to small pieces. Three hundred milligrams of silk cocoon were added into 3 mL of 6 different solvents including acetone (99.8%), ethyl acetate (99.5%), dimethyl sulfoxide (99.8%), ethanol (99.9%), methanol (99.9%), and purified water. Then, the mixture was shaken and incubated at 25 °C and 50 °C, 1400 rpm for 1 h. Each type of silk cocoon was repeatedly extracted in each solvent for 4 times. Then, all extracts were collected and centrifuged at 14,000 rpm for 5 min. The components in the extracts were identified by liquid chromatography–mass spectrometry (LC-MS).

2.2.2. Optimization of flavonoid glycoside hydrolysis

Rutin (quercetin-3-*O*-rutinoside) was used as a flavonoid glycoside in this study. Rutin (0.1% in ethanol) was hydrolyzed with 2 different acids, sulfuric acid and hydrochloric acid, at 0.125, 0.25, 0.5, 1, and 2 M of final concentration. After that, the mixture was shaken and diluted in the mixture of purified water:ethanol (1:10). Finally, the mixture was incubated at 75 °C, 1400 rpm for 1 h, centrifuged at 14,000 rpm for 5 min, and analyzed by liquid chromatography–ultraviolet detection LC-UV. The degradation of rutin and the amount of quercetin were calculated by peak area.

2.2.3. Quantification of flavonoids in silk cocoons

Silk cocoons were cut to small pieces. Three hundred milligrams of silk cocoon were added into 3 ml of 3 different solvents which were ethanol (99.9%), methanol (99.9%), and purified water. Then, the mixture was incubated at 50 °C, 1400 rpm for 1 h. Each type of silk cocoon was repeatedly extracted in each solvent for 4 times. Then, all extracts were collected. Then, the extracts were altogether. After that, sulfuric acid was added into the extract to be 2 M of sulfuric acid in final concentration. The mixture was shaken and diluted in the mixture of purified water: solvents (1:10). Finally, the mixture was incubated at 75 °C, 1400 rpm for 1 h. The extract was also centrifuged at 14,000 rpm for 5 min and analyzed by LC-MS. The amount of flavonoids in the extract was calculated by Progenesis QI software version 1.0 (Nonlinear Dynamics, Newcastle, UK).

The MS measurements were carried out on a Bruker maxis impact q-TOF (quadrupole-time-of-flight) instrument (Bruker Daltonics GmbH, Bremen, Germany). The preceding chromatographic separation was carried out on a Thermo Scientific/Dionex Ultimate 3000 high performance liquid chromatography (HPLC) on a 2.1 × 100.0 mm Agilent RRHD Zorbax C_{18} column (particle size 1.8 μm). Parameters of LC-MS for identifications and quantifications were shown in Table 1.

Table 1
Parameters of LC-MS for silk cocoon identification and quantification.

General parameters
LC - General parameters:
Solvent A: Water (MilliQ) + 0.01% formic acid
Solvent B: Acetonitrile (LC-MS grade; Fisher Scientific, Germany)
Gradient: 5%B to 100%B in 12 min
Column Temperature: 40 °C
Injection volume: 5 μL
Flow: 0.6 ml/min
MS - General parameters:
Scan Region: 50–800 m/z
Source Voltage: 4500 V with 500 V End-Plate Offset
Nebulizer gas was Nitrogen with a pressure of 3,0 bar
Dry gas flow (nitrogen) was set to 12 ml/min at a temperature of 200 °C
Parameters during identification and quantification
MS - Parameters during MS/MS (identification):
Three precursors have been analyzed per second alongside a full-scan, resulting in a cycle rate of 1.0 s.
MS - Parameters during full-scan (quantification):
Funnel radiofrequencies: 300 Vpp
Hexapole Radiofrequency: 50 Vpp
Quadrupol acceleration voltage: 5 eV
Low Mass Filter: 50 m/z
Pre-Pulse Storage: 6,0 μs
Transfer time: 50,0 μs
Collision Energy: 10,0 eV
Collision Radiofrequency: 500 V_{pp}
Scan Rate: 4 Hz

2.2.4. Evaluation of antioxidant activity of the flavonoids extracted

Silk cocoons were cut to small pieces. Three hundred milligrams of silk cocoon were added into 3 mL of 3 different solvents which were ethanol (99.9%), methanol (99.9%), and purified water. Then, the mixture was incubated at 50 °C, 1400 rpm for 1 h. Each type of silk cocoon was repeatedly extracted in each solvent for 4 times. Then, all extracts were collected. DPPH (0.3 mM) in ethanol was added into the extract. After that, the mixture was incubated in the dark room for 30 min. The absorbance of the mixture was measured using Biospectrometer (Eppendorf, Hamburg, Germany) at 517 nm. Trolox was used as antioxidant standard agent (Trolox calibration range $k = -0.004$, $d = 0.4224$, $r^2 = 0.9918$).

3. Results

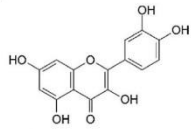
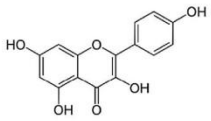
Three strains of silk cocoon (Chul 1/1; white cocoon, Chul 3/2; greenish cocoon, and Chul 4/2; yellow cocoon) were shown in Fig. 1.

3.1. Identification of flavonoids in silk cocoons

Quercetin and kaempferol data of the extract of Chul 3/2 silk cocoon extracted by methanol were shown in Table 2. LC-MS spectrum of the extract of Chul 3/2 silk cocoon extracted by methanol at 50 °C was shown in Fig. 2. Quercetin (peak 11) was detected around 5.2 min with 303.0503 m/z. This actual quercetin peak was confirmed by MSMS data at 153, 229, 137, 165, 257 m/z. Peak 2, 5, and 6 had protonated molecular and aglycone ions at 465 and 303 m/z, suggesting that it was a quercetin glycoside. Moreover, the results from MSMS showed that quercetin derivatives in the silk cocoon were only found in monosaccharide form at retention time 3.3, 3.9, and 4.1 min. These retention times were shorter than quercetin aglycone, implying that the compound might have a sugar at some position. Kaempferol (peak 12) was detected around 5.8 min with 287.0548 m/z. MSMS data at 153, 165, 121, 213, 258 m/z were used to confirm that it was the actual kaempferol peak. Kaempferol derivatives were also found in monosaccharide form at retention times 2.9, 3.4, 3.6, 4.3, 4.4, 4.5

Fig. 1. Silk cocoon strain (A) Chul 1/1; white cocoon (B) Chul 3/2; greenish cocoon (C) Chul 4/2; yellow cocoon.

Table 2
LC-MS data, protonated molecules (*m/z*) for peaks, the retention times (Rt), and MSMS fragments of the quercetin and kaempferol in the extract of Chul 3/2 silk cocoon extracted by methanol at 50 °C.

Flavonoids	Rt (min)	(M+H)+ (*m/z*)	MSMS (*m/z*) (RI %)	Fragment formula
Quercetin 	5.2	303.0503	303.0502 (100), 153.0178 (21.6), 229.0489 (21.1), 137.0231 (20.5), 165.0180 (14.8), 257.0437(13.8)	$[C_{15}H_{10}O_7+H]+$ $[C_7H_4O_4+H]+$ $[C_{13}H_7O_4+2H]+$ $[C_7H_4O_3+H]+$ $[C_6H_5O_4]+$ $[C_{14}H_8O_5+H]+$
Kaempferol 	5.8	287.0548	287.0546 (100), 153.0179(23.7), 165.0178(17.7), 121.0280 (15.5), 213.0536 (7.7)	$[C_{15}H_{10}O_6+H]+$ $[C_7H_4O_4+H]+$ $[C_6H_5O_4]+$ $[C_7H_5O_2]+$ $[C_{13}H_7O_3+2H]+$

and 4.6 min with 449 and 287 m/z (peak 1, 3,4,7–10). These retention times were shorter than kaempferol aglycone, implying that the compound might have a sugar at some position.

The quercetin and kaempferol were not found in all types of cocoon extracted by acetone and ethyl acetate at 25 °C and 50 °C. For DMSO and methanol extraction at 25 °C and 50 °C, quercetin, quercetin derivative, kaempferol, and kaempferol derivative were identified in Chul 3/2 silk cocoon. In contrast, the quercetin and kaempferol peaks were not found in the extract of Chul 1/1 and Chul 4/2 silk cocoon. For ethanol extraction, quercetin, quercetin derivative, kaempferol, and kampferol derivative were identified only in the extract of Chul 3/2 silk cocoon extracted at 50 °C. However, in purified water extraction, quercetin and kaempferol derivatives were identified in Chul 3/2 silk cocoon both at 25 °C and 50 °C (Fig. 3).

3.2. The optimal condition of flavonoid glycoside hydrolysis

The optimal condition of flavonoid glycosides hydrolysis was reported in this study. Rutin (quercetin-3-*O*-rutinoside) as a quercetin glycoside was hydrolyzed to measure amount of pure quercetin. Sulfuric acid and hydrochloric acid were used as hydrolyzing agent. The concentrations of rutin after hydrolysis with sulfuric acid and hydrochloric acid continuously decreased following the accumulation of acid while the concentrations of quercetin continuously increased (Fig. 4). Comparing between 2 different acids hydrolysis, the concentration of quercetin after hydrolysis with sulfuric acid was higher than that of hydrochloric acid. The highest quercetin concentration was found when hydrolyzed with 2 M of acids at final concentration. Therefore, the optimal acid for flavonoid glycoside hydrolysis was 2 M of sulfuric acid at final concentration.

3.3. Amounts of flavonoids in silk cocoons

The amounts of quercetin and kaempferol were highest in the extract of Chul 3/2 silk cocoon extracted by purified water comparing with Chul 1/1 and 4/2 (Table 3). Moreover, methanol and ethanol were found to extract the higher amounts of quercetin and kaempferol from Chul 3/2 silk cocoon than Chul 1/1 and 4/2. Nonetheless, there was no quercetin and kaempferol detected in any extract of Chul 1/1 and Chul 4/2 silk cocoon extracted by any solvents. Comparing among different solvents for Chul 3/2 silk cocoon extraction, purified water could extract the higher amount

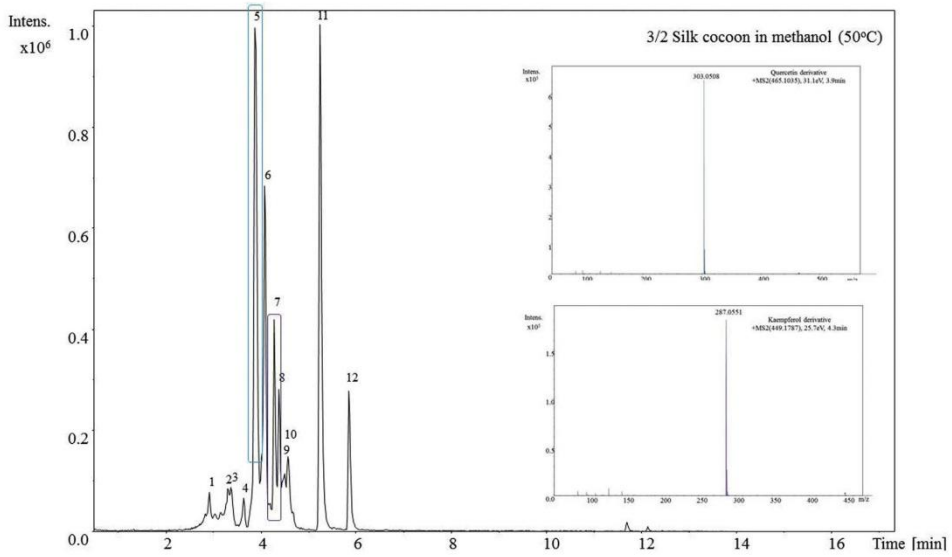


Fig. 2. LC-MS spectrum of the extract of Chul 3/2 silk cocoon extracted by methanol at 50 °C (1, 3, 4, 7–10: kaempferol derivative; 2, 5, 6: quercetin derivative; 11: quercetin; 12: kaempferol) (Isolation mass at 303.0505; 325.0324; 465.0505; 487.0524; 287.0555; 309.0375; 449.0553; 471.0372 ± 0.01) and MSMS spectrum of quercetin and kaempferol derivatives of the extract of Chul 3/2 silk cocoon extracted by methanol at 50 °C.

of quercetin and kaempferol than the methanol and ethanol. Methanol seemed to be better than ethanol for extraction (see Table 4).

3.4. Antioxidant activity of silk cocoons

The antioxidant activity of the flavonoids extracted from different strains of silk cocoon was shown in Fig. 5. The extract of Chul 3/2 silk cocoon extracted by purified water showed the highest antioxidant activity (101 μmole of trolox equivalence/1 g of dried cocoon). Comparing among different solvents in each strain of silk cocoon, the purified water extract showed the highest antioxidant activities, followed by methanol and ethanol extracts, respectively. Comparing among different strains of cocoon in each solvent, antioxidant activities of the extract of Chul 3/2 silk cocoon were higher than those of Chul 1/1 and Chul 4/2 silk cocoon. Antioxidant activities in purified water of Chul 1/1 silk cocoon were also higher than Chul 4/2 silk cocoon. However, antioxidant activities in ethanol and methanol solvents of Chul 1/1 and Chul 4/2 silk cocoon were not difference (see Fig. 6).

4. Discussion

Silk cocoon in each strain has different colors. The color of silk cocoon is a result of the pigment components. The major components of these pigments are flavonoid and carotenoid. Flavonoids in silk cocoon have function to protect the worm inside from outer environment including microorganism protection and UV shielding [3,9]. It was reported that different food of larvae (mulberry leaves) and silk worm strain may affect on the amount and activity of flavonoids [2,3]. In this study, flavonoids in 3 strains of silk cocoon extracted by 6 solvents were identified. The results demonstrated that quercetin, quercetin derivative, kaempferol, and kaempferol derivative were identified in Chul 3/2 silk cocoon (greenish cocoon). Because of some flavonoid was closely mass spectrum such as luteolin (287.0555 m/z) and kaempferol (287.0556 m/z). However the MSMS of luteolin were shown at 269, 241, 153, 137, 135 m/z while the MSMS of kaempferol were shown at 269, 241, 213, 259, 231,165, 153, 121, 133 m/z [10]. Moreover, luteolin and kaempferol have totally different retention times. On C18 column with the gradient that was used in this study, the retention time of luteolin was shorter than kaemoferol around 0.5–0.6 min. Therefore, the MSMS fragments in this study could confirm that the flavonoids inside the silk cocoon were quercetin and kaempferol (Table 2). Additionally, the quercetin and kaempferol derivatives were also confirmed by MSMS fragments that they were composed of monosaccharide (Fig. 2). These results agreed with the results of Kurioka et al. They found that yellow green cocoon (Sasamayu) contained 3 quercetin glycosides and 2 kaempferol glycosides [3]. Lin et al. reported that quercetin and kaempferol (M+H)+ were identified at 303 and 287 m/z respectively [11]. Quercetin glycoside also contained 465 and 303 m/z. Kaempferol glycoside was found at 449 and 287 m/z [11,12]. Quercetin and kaempferol were also identified in greenish cocoon (Daizo) of methanol extraction [13]. However, flavonoids in Chul 1/1 and Chul 4/2 silk cocoon in this study could not identify, the flavonoid inside that types of silk cocoon may be too low for identification. The previous study also reported that the color of greenish cocoon is mostly from flavonoid glycosides while the color of yellow is mostly from carotenoids [14,15] but white cocoon did not contain pigments. For solvent extraction, obvious peak of flavonoids was found in DMSO, ethanol, methanol, and purified water extract but they could not identify in

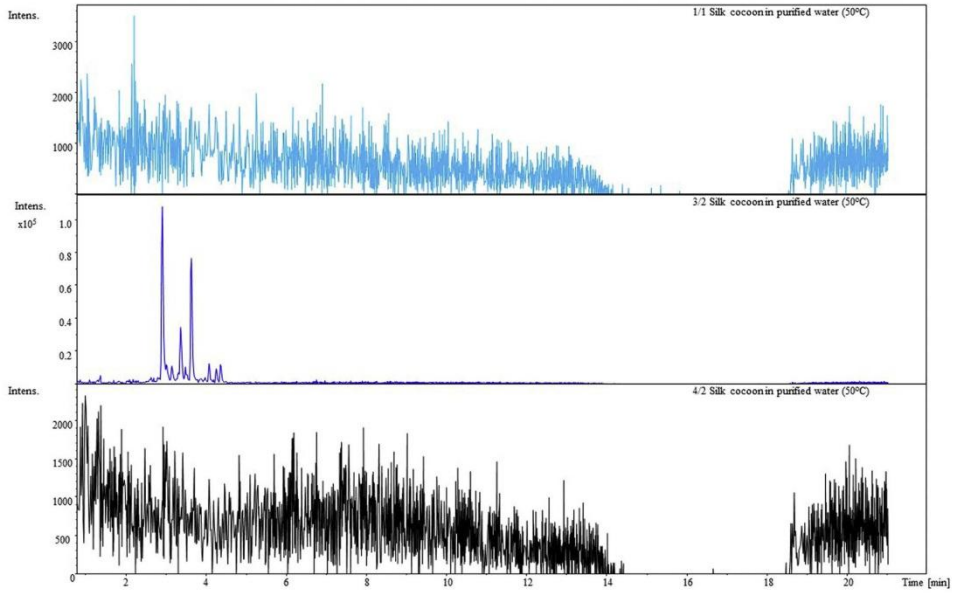


Fig. 3. LC-MS spectrum of the extracts of Chul 1/1, Chul 3/2, and Chul 4/2 silk cocoon extracted by purified water at 50 °C (Isolation mass at 303.0505; 325.0324; 465.0505; 487.0524; 287.0555; 309.0375; 449.0553; 471.0372 ± 0.01).

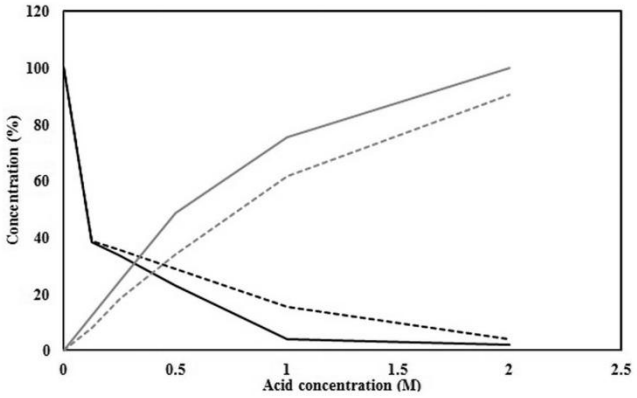


Fig. 4. Rutin and quercetin concentration (%) after hydrolysis with 0.125, 0.25, 0.5, 1, and 2 M of sulfuric acid and hydrochloric acid (black line = rutin concentration; grey line = quercetin concentration; solid line = sulfuric acid hydrolysis; dash line = hydrochloric hydrolysis).

acetone and ethyl acetate extract owing to polar structure of flavonoids [5]. Temperature also had an effect on flavonoid extraction. In ethanol extraction, higher temperature (50^{O}C) showed the peak of quercetin and kaempferol but there were no obvious peaks of flavonoids in 25^{O}C. These results conformed with Zhao et al. They found that higher temperature extract was effective than lower temperature [13].

For the quantification flavonoids of silk cocoon, 2 M of sulfuric acid was the optimizing hydrolysis agent that could hydrolyze flavonoid glycoside to aglycone (Fig. 4). This result conformed with

Table 3
LC-MS data, identified flavonoids in the extract of Chul 1/1, 3/2, and 4/2 silk cocoon extracted by 6 solvents at 25 °C, 50 °C.

Solvent	Silk cocoon types					
	Chul 1/1		Chul 3/2		Chul4/2	
	25 °C	50 °C	25 °C	50 °C	25 °C	50 °C
Acetone	NF	NF	NF	NF	NF	NF
Ethyl acetate	NF	NF	NF	NF	NF	NF
DMSO	NF	NF	Quercetin Quercetin derivative Kaempferol Kaempferol derivative	Quercetin Quercetin derivative Kaempferol Kaempferol derivative	NF	NF
Methanol	NF	NF	Quercetin Quercetin derivative Kaempferol Kaempferol derivative	Quercetin Quercetin derivative Kaempferol Kaempferol derivative	NF	NF
Ethanol	NF	NF	NF	Quercetin Quercetin derivative Kaempferol Kaempferol derivative	NF	NF
Water	NF	NF	Quercetin derivative Kaempferol derivative	Quercetin derivative Kaempferol derivative	NF	NF

*NF; not found.

Table 4
Quercetin and kaempferol concentration (μg/1 g of dried silk cocoon) in the extract of silk cocoons (Chul 3/2 silk cocoon) extracted by ethanol, methanol, and purified water.

Type of silk cocoon	Solvent	Quercetin (μg/g)	Kaempferol (μg/g)
Chul 3/2	Ethanol	0.3811 ± 0.0080	0.0346 ± 0.0025
	Methanol	20.2055 ± 0.2268	2.5491 ± 0.00313
	Purified water	23.6336 ± 0.2529	5.2077 ± 0.1124

previous study that 2 M of sulfuric acid could hydrolyze quercetin glycoside to quercetin [16]. The amount of flavonoids, Chul 3/2 silk cocoon showed highest amounts of flavonoids and purified water was the best solvent for silk cocoon extraction (Table 3). Methanol was the other choice for silk cocoon extraction but the amounts of flavonoids may be rarely found in ethanol extraction. These results agreed with Wang et al. They found that greenish cocoon (Daizo) contained the highest amount of flavonoids. The white cocoon contained the lowest amount of flavonoids [17]. Butkhup et al.

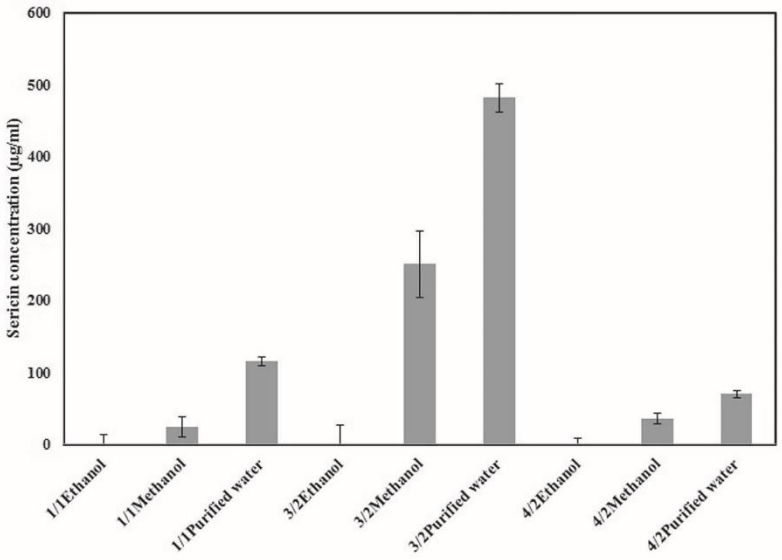


Fig. 5. Sericin concentration (μg/ml) of the extract of silk cocoons (Chul 1/1, Chul 3/2, Chul 4/2 silk cocoon type) extracted by ethanol, methanol, and purified water.

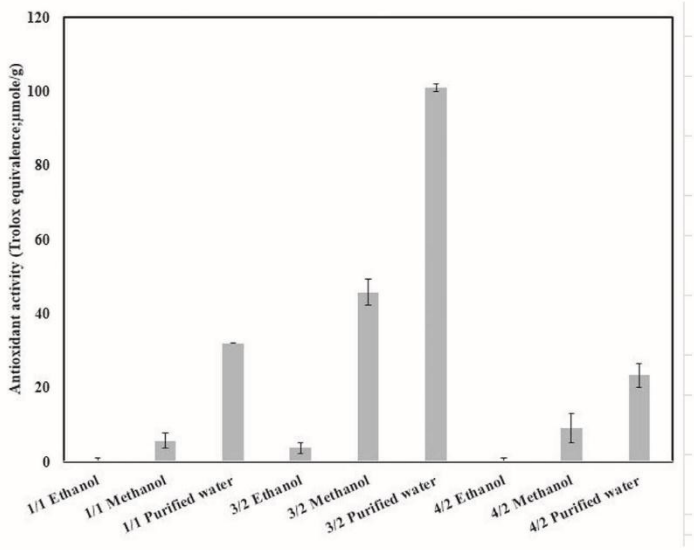


Fig. 6. Antioxidant activity (trolox equivalence (µmole/1 g of dried cocoon)) of the extract of silk cocoons (Chul 1/1, Chul 3/2, Chul 4/2 silk cocoon type) extracted by ethanol, methanol, and purified water.

reported that total amounts of flavonoids of silk cocoon in water extract were significantly higher than that in ethanol extract because of many polar hydroxyl groups within compounds [5]. Prommuak et al. also revealed that the amounts of flavonoids of cocoon (Nangnoi) in water extract were higher than ethanol extract [7]. Moreover, these results also agreed with Wach et al. They confirmed that amounts of quercetin in water extraction was higher than methanol and ethanol extraction, respectively [18]. Zhao et al. also presented that the amounts of flavonoids in methanol with hydrochloric acid and water extract were higher than ethanol and acetone with hydrochloric acid and water extract [13].

The antioxidant activity in Chul 3/2 silk cocoon was higher than other types (Fig. 5) in this study. It may be result of higher flavonoids that contained in Chul 3/2 silk cocoon. This results conformed with Aramwit et al. Chul 3/2 silk cocoon extract with pigments in urea showed the highest tyrosinase activity (tyrosinase; enzyme that can catalyze the ortho-hydroxylation of monophenols to catechols and their subsequent oxidation to *ortho*-qunones) among other types (Chul 1/1 and Chul 4/2 silk cocoon) [19]. The antioxidant activity of cocoon (Nangnoi) with pigment was higher than cocoon extract without pigment [7,17]. Moreover, the antioxidant activities of silk cocoon in water extract in this study were higher than methanol and ethanol extract, respectively. Butkhup et al. reported that antioxidant activity of both yellow and white silk cocoon in water extract was higher than that in ethanol extract. Moreover, they found that phenolic compounds that included other substances had an effect on increased antioxidant activity [5]. From Table 3, there were almost no flavonoids in silk cocoon type Chul 1/1 and Chul 4/2 extraction but there was antioxidant activity (Fig. 5). For antioxidant activity test, all substances including flavonoids and other substances such as sericin or phenolic compound that had an antioxidant activity were extracted. Therefore, other substances may have an effect on these results. Aramwit et al. found that water extraction with heat could extract high amount of sericin from silk cocoon [20]. The sericin also contained potent antioxidant activity [21–23].

5. Conclusion

Three types of silk cocoon were extracted in 6 solvents. The highest amount of quercetin and kaempferol was found in Chul 3/2 silk cocoon. Purified water was the solvent that could extract the highest amount of quercetin and kaempferol. Moreover, antioxidant activity of Chul 3/2 silk cocoon in purified water was also higher than methanol and ethanol. No quercetin and kaempferol were identified in acetone and ethyl acetate extraction. Quercetin and kaempferol in Chul 1/1 and Chul 4/2 silk cocoon in all solvents were too low for this identification. However, Chul 1/1 and Chul 4/2 silk cocoon had still contained antioxidant activity.

Conflicts of interest

None.

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