ผลของสารสกัดเหงือกปลาหมอดอกขาวร่วมกับคอลลาเจนสแคฟโฟลด์ต่อการเกิดหลอดเลือด
ใหม่และการปิดของแผลในโมเดลผิวหนังของหนูไมซ์

นางสาวจุฑามาศ สมชัยชนะ

EFFECT OF ACANTHUS EBRACTEATUS VAHL. EXTRACT

IN COMBINATION WITH COLLAGEN SCAFFOLD ON ANGIOGENESIS

AND WOUND CLOSURE IN MICE SKIN MODEL

Miss Jutamard Somchaichana

A Thesis Submitted in Partial Fulfillment of the Requirements

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Thesis Title	EFFECT OF ACANTHUS EBRACTEATUS VAHL. EXTRACT IN COMBINATION WITH COLLAGEN SCAFFOLD ON ANGIOGENESIS AND WOUND CLOSURE IN MICE SKIN MODEL
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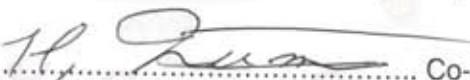
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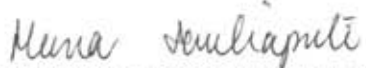
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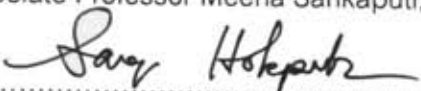
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จุฑามาศ สมชัยชนะ : ผลของสารสกัดเหงือกปลาหมอดอกขาวร่วมกับคอลลาเจนสแคฟโฟลด์ต่อการเกิดหลอดเลือดใหม่ และการปิดของแผลในโมเดลผิวหนังของหนูไมซ์ (EFFECT OF ACANTHUS EBRACTEATUS VAHL. EXTRACT IN COMBINATION WITH COLLAGEN SCAFFOLD ON ANGIOGENESIS AND WOUND CLOSURE IN MICE SKIN MODEL) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.ดร.สุทธิลักษณ์ ปทุมราช, Dr. Hideyuki Niimi และ ผศ.นพ.ถนอม บรรณ ประเสริฐ 72 หน้า.

ศึกษาผลของสารสกัดเหงือกปลาหมอที่ใช้ร่วมกับคอลลาเจนสแคฟโฟด์ในการหายของแผล โดยนำหนูไมซ์ ชนิด Balb/c mice (น้ำหนัก: 22-25 กรัม) ทำการสลบด้วย sodium thiopental และทำให้เกิดบาดแผลชนิด full thickness wound ด้วยการใช้กรรไกรตัดผิวหนังบริเวณด้านหลังเป็นขนาด 1.5×1.5 ตารางเซนติเมตร จากนั้นทำการ ปลูกถ่าย collagen sheet ที่บริเวณแผล สัตว์ทดลองจะถูกแยกออกเป็น 9 กลุ่ม คือ กลุ่มที่ไม่ทำให้เกิดแผลและกลุ่มที่มี แผลซึ่งแบ่งเป็นกลุ่มย่อยคือกลุ่มที่ให้น้ำเกลือ กลุ่มที่ได้รับการทาสารสกัดเหงือกปลาหมอในที่แผลปริมาณ 0.03, 0.3 และ 3 กรัมต่อกิโลกรัมน้ำหนักตัว กลุ่มที่ได้รับการปลูกถ่ายคอลลาเจนสแคฟโฟด์เพียงอย่างเดียว และกลุ่มที่ได้รับการ ปลูกถ่ายคอลลาเจนสแคฟโฟด์ร่วมกับสารสกัดเหงือกปลาหมอปริมาณ 0.03, 0.3 และ 3 กรัมต่อกิโลกรัมน้ำหนักตัว ใน วันที่ 3, 7 และ 14 หลังการเกิดแผลทำการวัดพื้นที่แผลด้วยโปรแกรม Image Pro-Plus วันที่ 3 จะทำการเก็บชิ้นเนื้อ เพื่อย้อม H&E ดูการบุกรุกของนิวโทรฟิวส์ทำการตรวจหาการเกิดหลอดเลือดใหม่ด้วย intravital fluorescence microscopy และวัดปริมาณหลอดเลือดฝ่อยในวันที่ 7 และ 14 นำชิ้นเนื้อตัวอย่างไปย้อม H&E ดูการเกิดของ re-epithelialization และตรวจหาปริมาณของVEGF โดยการทำ ELISA ผลการทดลองพบว่า 1) กลุ่มที่ทำการปลูกถ่ายคอล ลาเจนสแคฟโฟด์นั้น มีการบุกรุกของนิวโทรฟิวส์ที่มากกว่าอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่ให้น้ำเกลือ (p=0.03) แต่ในกลุ่มที่รับการปลูกถ่ายคอลลาเจนสแคฟโฟด์ร่วมกับการให้สารสกัดเหงือกปลาหมอ 0.3 กรัมต่อกิโลกรัมน้ำหนักตัว พบว่ามีการลดลงของปริมาณนิวโทรฟิวส์ที่ลดลงอย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่ทำการปลูกถ่ายคอลลาเจนสแคฟ โฟด์ (p=0.02) 2) ในวันที่ 14 แผลของกลุ่มที่ทำการให้การรักษาทุกกลุ่มมีการเพิ่มขึ้นของการปิดของแผลเมื่อเทียบ กับกลุ่มที่ให้น้ำเกลืออย่างมีนัยสำคัญ 3) การเกิด re-epithelialization ในกลุ่มที่ทาสารสกัดเหงือกปลาหมอ 0.03 กรัม ต่อกิโลกรัมน้ำหนักตัว และกลุ่มที่ปลูกถ่ายคอลลาเจนสแคฟโฟด์ร่วมกับการทาสารสกัดเหงือกปลาหมอ 0.3 กรัมต่อ กิโลกรัมน้ำหนักตัว มีการเพิ่มขึ้นของการเกิด re-epithelialization อย่างมีนัยสำคัญเมื่อเทียบกับกลุ่มที่ให้น้ำเกลือ (p=0.05, p=0.05, ตามลำดับ) 4) กลุ่มที่ทาสารสกัดเหงือกปลาหมอ 0.03 กรัมต่อกิโลกรัมน้ำหนักตัวและปลูกถ่ายคอล ลาเจนสแคฟโฟด์ร่วมกับการทาสารสกัดเหงือกปลาหมอ 0.3 กรัมต่อกิโลกรัมน้ำหนักตัว พบว่ามีการเพิ่มขึ้นของการเกิด หลอดเลือดใหม่อย่างมีนัยสำคัญต่อกลุ่มที่ให้น้ำเกลือในวันที่ 7 5) การวัดปริมาณของ VEGF พบว่ากลุ่มที่ ปลูกถ่ายคอล ลาเจนสแคฟโฟด์ร่วมกับการทาสารสกัดเหงือกปลาหมอ 0.3 กรัมต่อกิโลกรัมน้ำหนักตัว มีปริมาณ VEGF ที่เพิ่มขึ้น จาก ผลการทดลองจึงสามารถสรุปได้ว่า การปลูกถ่ายคอลลาเจนสแคฟโฟด์ร่วมกับการให้สารสกัดเหงือกปลาหมอ 0.3 กรัม ต่อกิโลกรัมน้ำหนักตัว นั้นมีผลต่อการลดการอักเสบเมื่อทำการปลูกถ่ายคอลลาเจน กระตุ้นการเกิดกระบวนการ re-epithelialization และการเกิดหลอดเลือดใหม่ที่ผิวหนังซึ่งส่งผลต่อการปิดของแผลที่เร็วขึ้น

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KEYWORDS: ACANTHUS EBRACTEATUS VAHL. / ANGIOGENESIS / COLLAGEN SCAFFOLD / WOUND CLOSURE.

JUTAMARD SOMCHAICHANA: EFFECT OF ACANTHUS EBRACTEATUS VAHL. EXTRACT IN COMBINATION WITH COLLAGEN SCAFFOLD ON ANGIOGENESIS AND WOUND CLOSURE IN MICE SKIN MODEL. ADVISOR: ASSOC. PROF. SUTHILUK PATUMRAJ, Ph.D, CO-ADVISOR: PROF. HIDEYUKI NIIMI, Ph.D., ASST. PROF. TANOM BUNAPRASERT, M.D. 72 pp.

This research was to study the effects of *Acanthus ebracteatus* Vahl. (AE) extract on the efficacy of collagen scaffold for wound healing. Balb/c mice (22-25 g) were used for this study. Animals were anesthetized with sodium thiopental (20µg/ml, intraperitoneally). The dorsal skin was cut with 1.5×1.5 cm^2 size, in order to develop a full-thickness wound. Wounded animals were divided into 8 groups: wound treated with normal saline (W+NSS), wound treated with AE, 0.03, 0.3 and 3 g/kg bw, (W+AE0.03, W+AE0.3, and W+AE3), wound implanted with bovine collagen scaffold (W+Coll), and W+Coll treated with AE at 3 doses (W+Coll+AE0.03, W+Coll+AE0.3, and W+ Coll+AE3). On day 3, 7 and 14 post-wound-operation, the wound area was measured using Digital Image analysis (Image Pro-Plus, Media Cybernetics, Inc). On day 3, the number of neutrophil infiltration was counted using H&E staining. On day 7 and 14, the angiogenesis in the wound area of each group was examined using intravital fluorescence microscopy and represented by percentage of capillary vascularity (%CV). The re-epithelialization and the level of VEGF were analyzed from wound tissue samples using H&E staining and ELISA, respectively. The results showed that: 1) number of neutrophil infiltration in W+Coll group increased significantly as compared to W+NSS (p=0.03), but in W+Coll+AE0.3, it decreased significantly as compared to W+Coll (p=0.02). 2) On day 14, the wound closure of all groups increased significantly as compared to W+NSS. 3) Re-epithelialization in W+AE0.03 and W+Coll+AE0.3 increased significantly as compared to W+NSS (p=0.05). 4) In W+AE0.03 and W+Coll+AE0.3, the % CV increased significantly as compared to W+NSS (p=0.001, p=0.03). 5) On day 7, VEGF level increased in W+Coll+AE0.3, significantly as compared to W+Coll (p=0.01). The combined treatment of *Acanthus ebracteatus* Vahl. extract (0.3 g/kg bw) with bovine collagen scaffold has shown the most benefit effects on wound management, which were attributed through the reduction of neutrophil infiltration, enhanced re-epithelialization and neocapillarization, and finally lead to rapid wound closure.

Field of Study : ....Physiology............   Student's Signature Jutamard Somchaichana

Academic Year : ....2008.............   Advisor's Signature ....

Co-Advisor's Signature ....

Co-Advisor's Signature ....

ACKNOWLEDGEMENTS

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LIST OF ABBREVIATIONS

VEGF	=	Vascular endothelial growth factor
FGFs	=	Fibroblast growth factors
EGF	=	Epidermal growth factor
KGFs	=	Keratinocyte growth factors
TGF-β	=	Transforming growth factor-β
IL-1	=	Interleukin-1
TNF	=	Tumour necrosis factor
HGF	=	Hepatocyte growth factor
IGF-1	=	Insulin-like growth factor-1
G-CSF	=	Granulocyte-colony stimulating factor
GM-CSF	=	Granulocyte macrophage-colony stimulating factor
PDGF	=	Platelet-derived growth factor
ECM	=	Extracellular matrix
HIF1-α	=	Hypoxia inducible factor 1- α
MMP	=	Metalloproteinases
TIMP	=	Tissue inhibitors of metalloproteinases
CV	=	Capillary vascularity
AE	=	*Acanthus ebracteatus* Vahl.

CHAPTER I

INTRODUCTION

A wound is a type of physical trauma. In the pathology, it specifically refers to a sharp injury which damages the dermis of the skin. There is a simple classification for wound into two types: open and closed wounds. The open wound is produced when the skin is torn, cut or punctured, while the closed wound is produced when a contusion is caused by its blunt force.

In general, open wound care needs to accomplish the following steps: 1) to stop dangerous blood loss, 2) to prevent infection, and 3) to promote healing.

For a case of deep wound, which is "full-thickness wound" (Rivera and Spencer 2007), dermal regeneration frequently fails, resulting in scar formation and wound contraction. For clinical treatment of the full-thickness wound, several tissue-engineered skin substitutes have been developed. Among these tissue-engineered substitutes, collagen scaffold is most useful in synthesizing or regenerating wound tissue. Previous studies show that a collagen–base scaffold can reduce contraction of the wound area (or volume) during the healing process (Freyman et al. 2001). A number of problems of skin substitutes for full-thickness wounds remain unresolved in regard to scar formation, wound contraction, loss in adnexal structures and so on.

Since wound healing is a dynamic process involving many biochemical and biophysical factors, the knowledge of skin tissue-engineering is necessary for clinical application of acute full-thickness wound model (Lamme et al. 1998). For instance, mesenchyme-derived signals are essential for epithelial proliferation, skin morphogenesis, homeostasis and differentiation. Skin wound healing also requires "*angiogenesis*" for achieving wound closure or recovery. In fact, impairment of wound angiogenesis may request a long time-duration for wound healing (William and Vincent 2003). Furthermore, poor blood perfusion and inflammation may decrease the dynamical process of wound healing, which results in increasing scar formation (Eming et al. 2007). *Acanthus ebracteatus* Vahl. (AE) has been used as a therapeutic agent for various diseases such as skin disease (Manasomboon 2004).

The agent has activities of anti-inflammation, anti-oxidation and anti-bacteria (Kongcharoensuntorn et al. 2005). In addition, one of its bioactive components is beta-sitosterol (Moon et al. 1999) which is a factor of angiogenesis. Therefore, it is reasonable to suppose that AE may be effective for wound healing via angiogenesis, anti-bacterial infection, and anti-inflammation.

The present study was aimed to qualitatively examine the effect of AE extract in combination with collagen scaffold on angiogenesis and wound closure using a mouse model of skin full thickness wound.

Parts of the present study were presented at the 7th Asian Congress for Microcirculation, the 11th Annual Meeting Thai Society of Microcirculation and the 2nd Science Research Conference.

CHAPTER II

LITERATURE REVIEW

A. Structure of the skin

The skin is the largest organ of the body, capable of self-regeneration. It has three layers as shown in Figure 2.1.
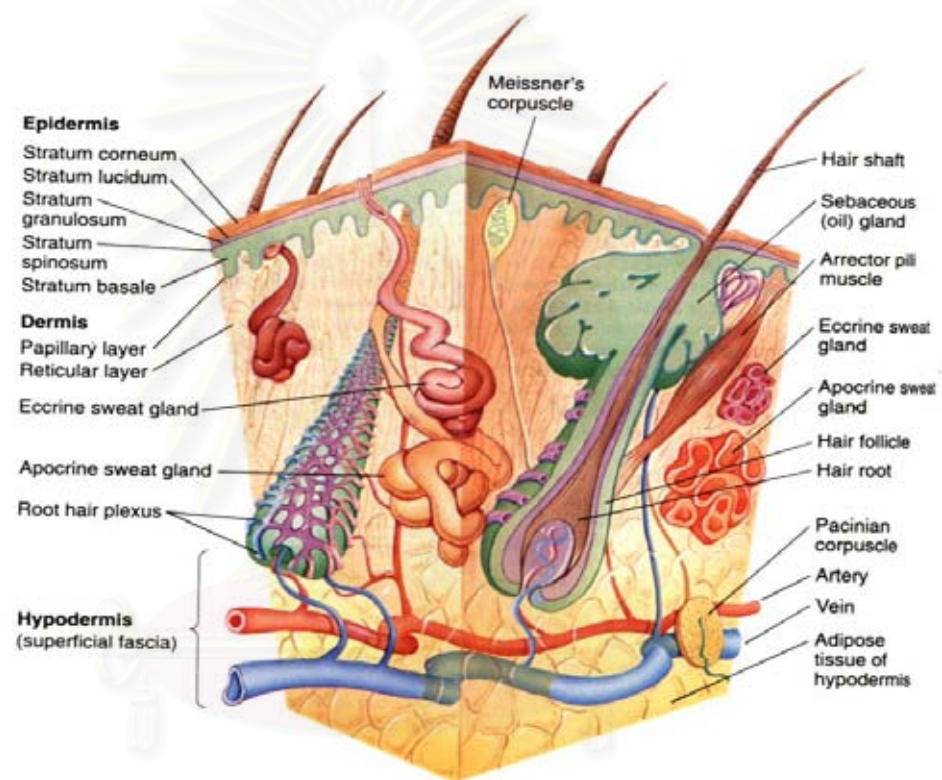
Figure 2.1 An illustration of the structure of the skin. The skin has three layers: epidermis dermis and subcutis or subcutaneous layer. [Copied from the website: (http://202.129.54.82/faculty/web_bed/apichat/cell-tissue/page/Skin.html)].

The epidermis is the upper or outer layers of the skin, is stratified squamous epithelium consisting primarily of keratinocytes in progressive stages of differentiation from deeper to more superficial layers. The epidermis has no blood vessels, so that it must receive

nutrients by diffusion from the underlying dermis through the basement membrane, which separates the layers. The epidermis contains the melanocytes, the Langerhans cells, Merkel cells and sensory nerves. The epidermis layer itself is made up of five sublayers that work together to continually rebuild the surface of the skin: stratum basale, stratum spinosum, stratum granulosum, stratum licidum, and stratum corneum (see Figure 2.1).

The dermis is the lower or inner layers of the skin. The more superficial is papillary dermis and the deeper is reticular dermis. The papillary dermis is thinner, consisting of loose connective tissue containing capillaries, elastic fibers, reticular fibers, and some collagen. The reticular dermis consists of a thicker layer of dense connective tissue containing larger blood vessels, closely interlaced elastic fibers, and coarse branching collagen fibers arranged in layers parallel to the surface. The reticular layer also contains fibroblasts, mast cells, nerve endings, lymphatics, and epidermal appendages.

The subcutis is the innermost layer of the skin, and consists of a network of fat and collagen cells. The subcutis is also known as the hypodermis or subcutaneous layer, and functions is conserving the body's heat, and as a shock-absorber, protecting the inner organs. It also stores fat as an energy reserve for the body. The blood vessels, nerves, lymph vessels, and hair follicles also cross through this layer.

B. Wound
1. Definition

A wound is a break in the epithelium of the skin and may be accompanied by disruption of the structure and function of normal tissue. Wounds are classified by characteristics of closure into open and closed wounds, or by the depth of tissue involvement into superficial, partial-thickness, full-thickness wounds.

2. Open wound

This can be classified into six different types, depending on the object that made the wound as follows:

Incisions or excised wounds – resulted from a clean, poignant object such as a knife, a razor or a glass sliver. Incisions which affect only the epidermis are classified as cuts, rather than wounds.

Puncture wounds - The skin is punctured or drilled by an object, such as a nail or needle.

Penetration wounds - resulted from an object such as a knife getting into the body.

Gunshot wounds - resulted from a bullet or similar projectile driving into or through the body.

Abrasions (grazes) - a superficial wound in which the topmost layer of the skin (the epidermis) is scraped off. Often caused by a sliding fall onto a rough surface.

Lacerations - Irregular wounds caused by a blunt impact to soft tissue which lies over hard tissue (e.g. laceration of the skin covering the skull) or tearing of skin and other tissues such as caused by childbirth.

3. Closed wound

These have fewer categories, but are just as dangerous as open wounds. The types of closed wounds are:

Contusions - (more commonly known as a bruise) - caused by blunt force trauma that damages tissue under the skin.

Hematoma - (also called a blood tumor) - caused by damage to a blood vessel that in turn causes blood to collect under the skin.

Crushing Injuries - caused by a great or extreme amount of force applied over a long period of time (www.wikipedia.org/wiki/Wound).

In open wound, it can be classified by the depth of tissue involvement, or other characteristics such as closure (primary or secondary intention). Wound depth is classified by the level of tissue destruction evident in the wound: *superficial, partial-thickness,* or *full-thickness.*

4. Wound deep

4.1 Superficial Wounds

When a wound is superficial, as is the case in most abrasions and blisters, only the epidermis is affected and has to be replaced. A truly superficial wound does not bleed and heals within a few days.

4.2 Partial-thickness wound

A partial-thickness wound does bleed, because the epidermis and part of the dermis are no longer present or have been affected. If left uncovered, a blood clot will cover the wound and a scab will form. The missing tissue will then be replaced, followed by regeneration of the epidermis. A partial-thickness wound can take from several days to several weeks to heal, depending on the patient and the wound treatments chosen.

4.3 Full-thickness wound

A full-thickness wound involves the epidermis and the dermis. The underlying fatty tissue, bones, muscles, or tendons may also be damaged. If full-thickness wounds cannot be sutured, the healing process will create new tissue to fill the wound, followed by regeneration of the epidermis. The full-thickness wound takes substantially longer to heal than does a partial-thickness wound, sometimes as long as several months. In fact, the mortality, though related to the total area of injury, is much more closely tied to the extent of full thickness destruction of skin (Burke et al. 1975).

C. Wound healing

Wound healing is a dynamic process. The immediate goal in repair is to achieve tissue integrity and homeostasis (Singer and Clark 1999). The process can be divided into four phases: hemeostasis, inflammation, proliferative and tissue remodeling (Bauer et al. 2005).
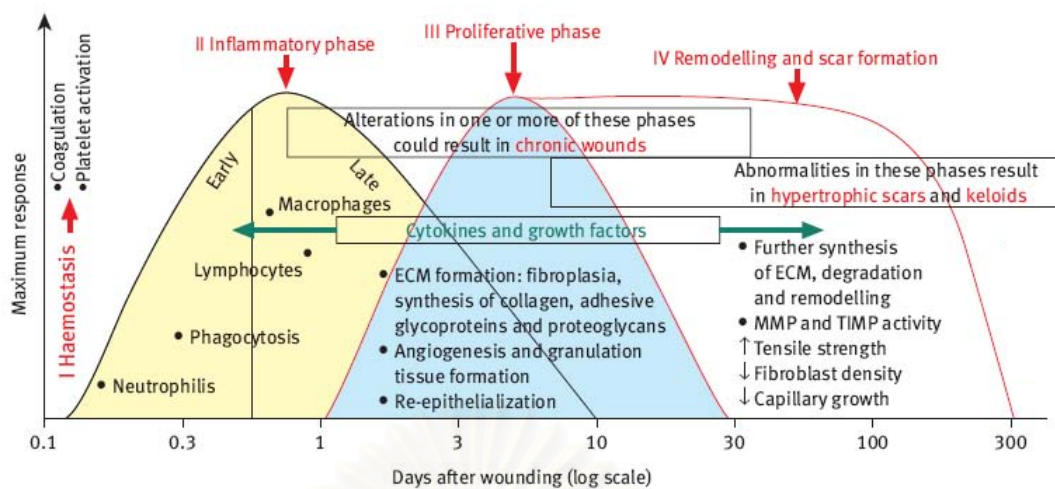
Figure 2.2 Phases of wound healing ECM: Extracellular matrix; MMP: Metalloproteinases; TIMP: Tissue inhibitors of metalloproteinases. Adapted from: Clark R A. In: Goldsmith L A (Editor): *Physiology, biochemistry, and molecular biology of the skin.* 2nd edition, Volume I. New York: Oxford University Press, 1991, p. 577.

1. Haemostasis (immediate)

    The microvascular injuries occur and extravasate of blood into the wound. The coagulation cascade and constriction of vessel walls; the resulting clot formation and platelet aggregation limits further blood loss (Figure 1.2). The platelets trapped in the clot are essential for haemostasis and a normal inflammatory response. In a recent report, the significance of platelets during tissue repair has been suggesting that platelets do not significantly affect the proliferative aspects of repair, including angiogenesis,collagen synthesis, and ultimately wound closure (Szpaderska et al. 2003). This process has many growth factors secrete such as:

- Platelet-derived growth factor (PDGF)

- Insulin-like growth factor-1 (IGF-1)

- Epidermal growth factor (EGF)

- Transforming growth factor-β (TGF-β)

- Platelet factor-IV

These proteins initiate the wound healing cascade by attracting and activating fibroblasts, endothelial cells and macrophages.

2. Inflammation (day 1 to day 3)

A few hours post injury, leukocytes infiltrate within the wound site. There is substantial evidence that the inflammatory response is instrumental to supplying growth factor and cytokines signals that orchestrate the cell and tissue movements (Simpson 1972; Leibovich and Ross 1975). Neutrophils normally begin arriving at the wound site within minutes of injury. The role of neutrophils is not only to be clearance contaminating bacteria, release a large variety of highly active antimicrobial substances (reactive oxygen species, cationic peptides, eicosanoids) and proteases [elastase, cathepsin G, proteinase 3 (PR-3), urokinase-type plasminogen activator (uPA)] (Weiss 1989) but neutrophils are also a source of pro-inflammatory cytokines, including interleukins 1 alpha and beta (IL-α and β) and tumor necrosis factor alpha (TNF-α) which provide some of the earliest signals activating local fibroblasts and keratinocytes (Hubner et al. 1996). In addition, there is increasing evidence that the various subsets of leukocytes have functions on vascular remodeling during repair. (Martin and Leibovich 2005; Eming et al. 2007). The neutrophils have been identified as an important source for pro-angiogenic factors, including VEGF-A and interleukin-8 (IL-8) (Ancelin et al. 2004; Li et al. 2003). Neutrophils are themselves phagocytosed by macrophages, which are present at the wound site within 2 days after injury. Macrophages are the most important cells in the later stages this process and regulatory cells for repair, which are phagocytic cells as well as produce growth factors factors, which are responsible to promote angiogenesis, cell proliferation and the synthesis of extracellular matrix (ECM) molecules such as:

- transforming growth factor-b (TGF-b)
- basic fibroblast growth factor (bFGF)
- platelet-derived growth factor (PDGF)
- vascular endothelial growth factor (VEGF)

3. Proliferation (day 3 to week 2)

The proliferative is characterized by fibroblast migration, deposition of the extracellular matrix and formation of granulation tissue. As granulation tissue forms keratinocytes at the wound edge synthesize proteases (MMP-1, stromolysin) and

components of the basal lamina (laminin-5) in order to re-epithelialize the wound. Epithelialization of the wound represents the final stage of the proliferative phase.

Fibroblast migration: 2–4 days after wounding, fibroblasts will appear in the wound and endothelial cells follow about one day later. The fibroblasts are attracted to the wound by many factors, including platelet-derived growth factor and transforming growth factor- β. The roles of fibroblasts within the wound are proliferation and producer the matrix proteins fibronectin, hyaluronan, collagen and proteoglycans. These components help to construct the new extracellular matrix, which is necessary for the repair process.

Collagens synthesis: is synthesized by fibroblasts. They provide strength and integrity to all tissues and so play a vital role in wound repair. Platelet-derived growth factor (Rhee and Grinnell 2006) basic fibroblast growth factor, transforming growth factor- β, interleukin-1, tumour necrosis factor induce collagen synthesis during the proliferative and remodeling phases.

Formation of granulation tissue: The granulation tissue that is indication of optimal healing is well established on day 3–5. Granulation tissue has a pink, soft, granular gross appearance. This phase is characterized by angiogenesis or formation of new blood vessels from pre-existing vessels at the site of injury (neovascularization). Several factors such as: vascular endothelial growth factor, platelet-derived growth factor, basic fibroblast growth factor and transforming growth factor-β induce angiogenesis. Granulation tissue bleeds easily if traumatized. The appearance of the granulation tissue may indicate by healing wounds, which have a moist and shiny, hyperaemic, and reddish appearance but it have an excessive, soft, friable wounds with a beefy-red color indicate that poor healing.

Angiogenesis: The process new blood vessels are formed. This formation occurs as endothelial cells lining blood vessels migrate into the surrounding collagen matrix. Agiogenesis is also an essential component of wound healing and of repair processes. (Knighton et al. 1990). Numerous inducers of angiogenesis have been identified, including the members of the vascular endothelial growth factor (VEGF) family, angiopoietins, transforming growth factors (TGF), platelet-derived growth factor, tumor necrosis factor-α, interleukins and the members of the fibroblast growth factor (FGF)

family (Marco et al. 2005). In addition, many factors control and influence angiogenesis including soluble growth factors, membrane-bound proteins, cell–matrix and cell–cell interactions, and many interacting systems.

Epithelialization: The epidermal cells migrate from the wound edges to wound area within a few hours of wounding. There is a marked increase in mitotic activity within the basal epithelial cells of the wound edges about 12 hours later. The differentiation of epithelial cell re-establishes the stratified epithelium. Once migration begins, the epidermal cells move one by one over the wound site until the wound is covered by a complete layer of cells. Epithelialization is modulated by several growth factors, including epidermal growth factor (EGF), keratinocyte growth factor (KGF), and transforming growth factor (TGF-α) may drive cell proliferation and wound closure (Saarialho-Kere et al. 1992).

4. Tissue remodeling and scar maturation (week 1 to several weeks)

The synthesis and remodeling of the extracellular matrix is initiated concurrently with the development of granulation tissue and continues over prolonged periods. There is continuous synthesis and breakdown of collagen as the extracellular matrix is constantly remodelled, equilibrating to a steady state about 21 days after wounding. Wound contraction occurs through the interactions between fibroblasts and the surrounding extracellular matrix and is influenced by a number of cytokines including transforming growth factor- β, platelet-derived growth factor and basic fibroblast growth factor.

Table 2.1 Cells involved in wound healing

Cell type	Function related to wound healing
Platelets	• Involved in thrombus formation • α-granules are a rich source of inflammatory mediators including cytokines (e.g. TGF- β, PDGF, β-thromboglobulin, platelet factor-4) • Major initial stimulus for inflammation
Neutrophils	• First cells to infiltrate site of injury • Phagocytosis and intracellular killing of invading bacteria
Monocytes (macrophages)	• Phagocytose and destroy invading bacteria • Clear debris and necrotic tissue • Rich source of inflammatory mediators including cytokines • Stimulate fibroblast division, collagen synthesis and angiogenesis
Lymphocytes	• Not clearly defined • May produce cytokines in certain types of wound
Fibroblasts	• Produce various components of the ECM, including collagen, fibronectin, hyaluronic acid, proteoglycans • Synthesize granulation tissue • Help to re-organize the 'provisional' ECM

TGF: Transforming growth factor; PDGF: Platelet-derived growth factor; ECM: Extracellular matrix (Stuart and David 2005).

Table 2.2. Growth factors involved in wound healing

Growth factor	Major source	Function related to wound healing
VEGF	Platelets, neutrophils	• Stimulates angiogenesis in granulation tissue • Stimulates formation of collateral blood vessels in peripheral vascular disease
FGFs	Fibroblasts, endothelial cells, smooth musclecells, macrophages; also brain, pituitary	• Proliferation of fibroblasts and epithelial cells; matrix deposition; wound contraction; angiogenesis • Accelerates formation of granulation tissue
KGFs	Fibroblasts	• Proliferation and migration of keratinocytes
EGF	Platelets, macrophages, keratinocytes; also and saliva, urine, milk, plasma	• Differentiation, proliferation, migration adhesion of keratinocytes • Formation of granulation tissue
PDGF	Platelets, fibroblasts, macrophages, endothelial cells	• Mitogenic for smooth muscle cells, endothelial cells and fibroblasts • Chemoattractant for neutrophils and fibroblasts • Fibroblast proliferation and collagen metabolism
G-CSF	Monocytes, fibroblasts, lymphocytes	• Stimulates production of neutrophils • Enhances function of neutrophils and monocytes • Promotes proliferation of keratinocytes
GM-CSF	Keratinocytes, macrophages, lymphocytes,fibroblasts	• Mediates proliferation of epidermal cells
TGF-α	Activated macrophages,	• Stimulates proliferation of

	epithelial cells platelets,	epithelial cells and fibroblast
		• Formation of granulation tissue
TGF- **β**	Platelets, macrophages,	• Mitogenic for smooth fibroblasts,
	Fibroblasts neutrophils,	muscle cells
	and keratinocytes	• Chemotactic for macrophages
		• Stimulates angiogenesis (indirect) and
		collagen metabolism
IL-1	Macrophages, lymphocytes,	• Neutrophil chemotaxis
	many other tissues and cells	• Fibroblast proliferation
TNF	Macrophages, mast cells,	• Fibroblast proliferation
	T-lymphocytes	
IGF-1	Fibroblasts, plasma, liver	• Fibroblast proliferation
		• Stimulates synthesis of sulphated
		proteoglycans and collagen
HGF	Fibroblasts, keratinocytes,	• Re-epithelialization
	endothelial cells, tumour	• Neovascularization
	cells	• Formation of granulation tissue

VEGF: Vascular endothelial growth factor; FGFs: Fibroblast growth factors; EGF: Epidermal growth factor; KGFs: Keratinocyte growth factors; TGF-β: Transforming growth factor-β; TGF-β: Transforming growth factor-β; IL-1: Interleukin-1; TNF: Tumour necrosis factor; HGF: Hepatocyte growth factor; IGF-1: Insulin-like growth factor-1; G-CSF: Granulocyte-colony stimulating factor; GM-CSF: Granulocyte macrophage-colony stimulating factor; PDGF: Platelet-derived growth factor (Stuart and David 2005).

D. Full-thickness skin wounds and scar formation

Unlike partial thickness wounds, full thickness wounds have no residual hair follicles from which new epithelium can grow. Instead, healing occurs as granulation tissue fills the space left behind as dead tissue is removed and the scar is formed (Van Rijswijk and Cuzzell 1991).

E. The events of wound contraction during full-thickness wound healing (Tanaka et al. 2004)

Initial phase (Fig. 2.3a): The wound expands temporarily due to inflammation. A small number of fibroblasts exist. Wound contraction does not occur.

Starting phase (Fig. 2.3b): The proliferation of granulation tissue including new blood capillaries, thin collagen fibers and edema starts. A few myofibroblasts appeared along the wound edge, and the contraction starts to occur, but weakly.

Increasing phase (Fig. 2.3c): The granulation tissue develops well with bundles of thin collagen fibers. A great number of myofibroblasts develop along the wound edge and wound bed. Myofibroblasts and collagen fibers form bridge-like structures across the wound and a ring around the wound edge. The wound contraction shows the strongest.

Decreasing phase (Fig. 2.3d): Myofibroblasts in the wound bed dramatically decrease and so the bridge-like structures disappear. The contraction weakens gradually.

Scar phase (Fig. 2.3e): Granulation tissue matures into scar tissue. Myofibroblasts are almost lost in the wound bed, and very few remain along the wound edge. The contraction is almost lost.
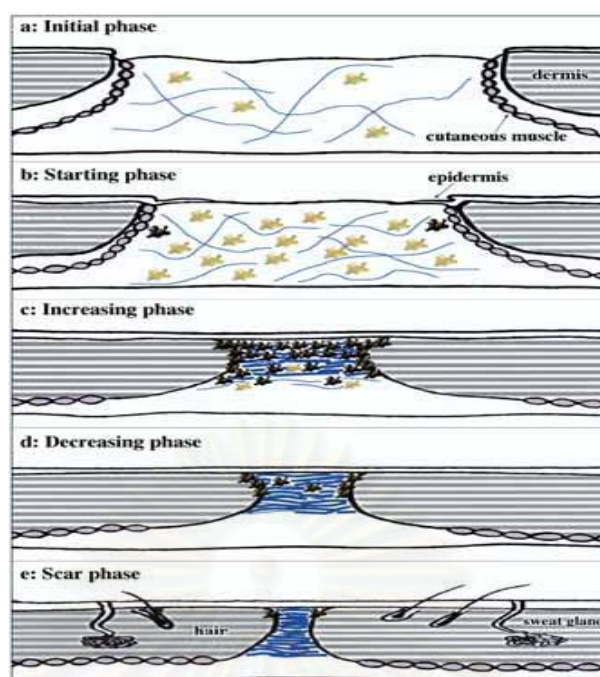
Figure 2.3 A consequence of events involved in the contraction of full-thickness wound.

Fibroblast.   Myofi broblast.   Collagen fibril.   Collagen fiber.

F. Efficacy endpoint of wound treatment (Guidance for Industry Chronic Cutaneous Ulcer and Burn Wounds (2006))

The two major outcomes: improve wound healing and improve wound care.

Improve wound healing:

- Incidence of complete wound closure is defined as skin re-epithelialization without drainage or dressing requirement confirmed at two consecutive study visits 2 weeks apart.

- Accelerate wound closure meaning a reduction in the time of healing using a time to event analysis (the event being complete closure).

- Facilitation of surgical wound closure.

- Quality of wound healing such as scarring, the contour and feel of healed skin, or normalization of skin markings or pigmentation.

Improve wound care:

- Treatment of wound infection

- Debridement

- Wound pain control

G. Evolution of tissue engineering in wound healing

Skin graft is a cut of skin that is removed from one area of the body to transplant the other area. The transplanted tissue is called a skin graft. Skin grafts may be recommended for very large wounds (full-thickness wound type), burns, venous ulcers, pressure ulcers, or diabetic ulcers which do not heal, surgeries, areas where there has been infection that caused a large amount of skin loss, cosmetic, skin cancer surgery. Skin grafts are often use after severe injuries when some of the body's skin is damaged. Surgical removal (excision or debridement) of the damaged skin is followed by skin grafting. The purposes of the grafting for reduce the time course of treatment needed and improve the function of body wound area. There are two types of skin grafts that are allograft and autograft.

Allograft is the common type of skin graft. A thin layer will be removed from a healthy part of the (body the donor section) or a full thickness skin graft, which involves pitching and cutting skin away from the donor section and implant on the wound. However, the transplanted allograft will be rejected transplanted organs depends upon activation of recipient T cells against (Hall 1991; Loveland et al. 1981; Loveland and McKenzie 1982).

Autograft Tissue transplanted from one part of the body to another in the same individual. The classical procedure for the coverage of full-thickness skin defects caused due to trauma or surgery is autologous skin grafting. However, poor skin quality and scar contracture occur frequently and are well-known problems in split skin grafted areas.

Collagen scaffold

Collagen is an important protein in animal kingdom. It is about 30% in the body of human (Prockop and Kivirikko 1995). In the normal tissue, collagen has the role of increasing strengthen and stabilize structure in human body. When injury occurs, body wants collagen for healing and returns to the normal function, however the collagen can cause tissue dysfunction. In contrast, less collagen will lead to incomplete healing.

Clinical studies have found that a collagen –base scaffold for skin regeneration reduces wound contraction during the healing process. These scaffolds should be biodegradable, but also stable enough to function as three-dimensional (3D) spacers. Furthermore, they should be biocompatible and support angiogenesis and neovascularization (Martin and Leibovich 2005).

In 1980, Yannas and Burke reported the basic design, the chemical composition, and the control of the pore structure of a collagen/glycosaminoglycan copolymer with a thin silicone membrane (pore ~70-200 µm) cover ---Integra artificial skin. The artificial dermis was published in 1988 in the management of acute burns immediate wound excision (Heimbach 1988). Integra has become a standard method of covering major burn wounds since the U.S. Food and Drug Administration granted the manufacturer a license in 1996 (Moiemen et al. 2006). Currently Integra is the most widely accepted synthetic skin substitute for use in burn patients to release of contracture, tight and keloid scar (Isabel et al. 2002; Frame et al. 2004). In addition, the advantage is to reduce donor site morbidity (faster healing with less scaring) and cross-infection (Eran et al. 2006).

H. Angiogenesis

Angiogenesis is defined by the formation of new blood vessels from pre-existing ones. It plays a role in wound repairment (Carmeliet and Peter 2000). Steps of angiogenesis begin with the degradation of the basement membrane by activated releasing protease endothelial cells that migrate and proliferate, leading to the formation of solid endothelial cell sprouts into the stromal space. Then, vascular loops are formed and capillary tubes are developed with formation of tight junctions and deposition of new basement membrane.

Angiogenesis can be induced by vascular endothelial growth factor (VEGF) family, angiopoietins (Ang), transforming growth factor-α and–β (TGF-α and -β), platelet-derived growth factor (PDGF), tumor necrosis factor-α (TNF-α), interleukins, chemokines, and the members of the fibroblast growth factor (FGF) family (Marco et al. 2005). The angiogenesis process is an essential for healing wounds for restoring blood flow to tissues after injury. Clinically, new capillaries first become visible in the wound

bed 3–5 days after injury, and their appearance is synonymous with granulation, the creation of a provisional matrix comprised of proliferating blood vessels, migrating fibroblasts and new collagen (Tonnesen et al. 2000).

Wound angiogenesis is amplified by inflammation. Macrophages and monocytes release myriad angiogenic factors as they marginate into the wound bed, including PDGF, VEGF, Ang-1, TGF-β, bFGF, interleukin- 8 (IL-8), and tumor necrosis factor alpha (TNF-α) (Richardson et al. 2001). Several growth factors (PDGF, VEGF, and bFGF) synergize in their ability to vascularize tissues (Amano et al. 2002). Expression of the inducible COX-2 enzyme during the inflammatory stage of healing also leads to VEGF production and other promoters of angiogenesis (Giordano and Johnson 2001).

The production of the vascular endothelial growth factor (VEGF) is strongly up-regulated in wound healing. It is secreted by activated macrophages and keratinocytes promoting new capillary formation within the wound bed. Impairment of new vessel formation results in low-quality wound healing due to poor blood perfusion (Tonnesen et al. 2000). Thus, it is our urgent task to increase vascularization for tissue regeneration and repair of chronic, non-healing ischemic wounds.

I. *Acanthus ebracteatus* Vahl.

Acanthus ebracteatus Vahl. belongs to a family of Acanthaceae. Thai name is ngueak plaa mo. It is mangrove and salt-marsh plant, an erect or reclining, up to 1 m tall, scarcely branched; leaves oblong, 12-20 cm × 3-5 cm. as shown in Figure 2.4. Parts of *Acanthus ebracteatus* Vahl. are used as therapeutic agent; seeds are used as anthelmintic. The juices from leaves is used to apply on the head to prevent hair loosing, anti-inflammatory, anti-asthmatic, longevity, shingle and skin disease (Manasomboon 2004).

Figure 2.4 *Acanthus ebracteatus* Vahl.

Bioactive compound in *Acanthus ebracteatus* Vahl.

Bioactive compounds isolated from *Acanthus ebracteatus* Vahl. such as flavonoids, isoverbascoside, acteoside, martynoside and lignans have anti-oxidant effect. The anti-inflammation is also from some bioactive compounds been reported to have shown in the Table 2.3 (Manasomboon 2004; Siripong et al. 1998). The report of Laupattarakasem et al. in 2003 showed that *Acanthus ebracteatus* Vahl. could exert anti-inflammatory effect by reducing thombaxane B_2 (TXB_2) and leukotriene B_4 (LTB_4). In 2005, Kongcharoensuntorn et al. demonstrated anti-bacterial and antioxidant activity in *Acanthus ebracteatus* Vahl. extract. In addition, β-sitosterol extracted from *Acanthus ebracteatus* Vahl. might act as angiogenic factor (Choi et al. 2002).

Beta-sitosterol

β–sitosterol is one of several phytosterols of which chemical structure similar to that of cholesterol. Even though the effect of β –sitosterol extract from *Acanthus ebracteatus* Vahl. has not been reported, β –sitosterol from Aloe vera has been indicated for its action on wound healing. For instance, Eun-Joung Moon.,et al.(1999) and Seongwon Choi., et al.(2002) reported angiogenic effect of β–sitosterol isolated from Aloe Vera gel. In their studies, by using Chorioallantoic membrane (CAM) assay, immunohistochemistry, western blotting and matrigel plug assay, it has been shown that β-sitosterol may increase the production of endothelial cell-mitogenic factors such as bFGF, aFGF and/or others whose activities were markedly enhanced by exogenous heparin. FGF were shown to play the role of stimulator of angiogenesis (Risau 1990) and mitogenic for several cell types present at the wound site, including fibroblasts, which synthesize collagen matrix and keratinocytes. β-sitosterol could also enhance migration *in vitro* model and enhance angiogenesis by increasing VEGF, VEGFR(Flk-1) and laminin.

In addition, β–sitosterol is reported to have effects on expression of transforming growth factor-β, stimulation of reepithelialization, granulation tissue formation and neovascularization (Werner et al. 2001) and the activity of Protein Kinese C-α (PKC- α) membrane in stromal cells of the prostate *in vitro* (Kassen et al. 2000). PKC- α is promoter angiogenic factor of human endothelial cells and inducer VEGF (Xu et al. 2008).

Table 2.3: Bioactive compounds in *Acanthus ebracteatus* Vahl.

#	Compounds	Biological effect/activation
1	Flavonoids	
	- Schaftoside	Anti-oxidant
	-Luteolin-7-O-**β**-D-glucuronide	Anti-oxidant, prevent of inflammation, promoter of carbohydrate metabolism and immune system.
2	Phenylpropanoids	
	- Verbascoside	Anti-inflammatory
	- **β**-hydroxyacteoside	Anti-bacterial
	- Isoverbascoside	Anti-oxidant, immunosuppressive
	-Acteoside	Anti-oxidant, Anti-bacterial, Anti-inflammatory, immunosuppressive
	- Martynoside	Anti-oxidant
3	Lignans	Anti-oxidant
4	**β**-sitosterol	Anti-inflammatory, angiogenic factor

CHAPTER III

MATERIALS AND METHODS

A. Chemical substances

We used the following substances:

- *Acanthus ebrecteatus* Vahl. Extract (from The Government Pharmaceutical Organization)
- Bovine collagen type I (from Sigma Chemical Co, USA)
- Fluorescein isothiocyanate (FITC)-labeled dextran, MW. 150,000 (FITC-dx-150) (from Sigma Chemical Co, USA)
- Thiopental (pentobarbital) (from Sigma Chemical Co, USA)

B. Experimental animals

Male Balb/c mice 8-10 weeks and weighing about 22-25 g were purchased from the National Laboratory Animal Center, Salaya Campus, Mahidol University, Thailand. They were taken to rest for a week after arrival at the Animal Center, Faculty of Medicine, Chulalongkorn University before use. The animals were kept in a pathogen limited room where temperature was controlled at 25±3°C. During the experiments the animal was housed one animal per cage with free access to sterilized water and standard laboratory chow with a 12 hours light and dark cycle.

C. Animal preparation

In this study the animals were divided randomly into two groups: sham and wound groups.

3.1 Sham group (Sham)

The animal was anesthetized with thiopental (pentobarbital) 20 µg/ml intraperitoneally. After shaving the skin hair, the animal was disinfected with a non iodine-containing alcohol. A square 1.5 cm. x1.5 cm was drawn on the dorsal skin, but the dorsal skin was not cut.

3.2 Wound group

Method of wound creation

The animal was anesthetized by thiopental (pentobarbital) 20 µg/ml intraperitoneally. After shaving the skin hair, the animal was disinfected with a non iodine-containing alcoholic skin disinfectant. Full-thickness skin defects of 2.25 cm^2 (1.5×1.5 cm) down to the muscle fascia resecting the panniculus carnosus were created with scissors corresponding to a defect size of about 10% Total Body Surface Area (Michael et al. 2000).

Wounded groups were divided into 8 subgroups:

3.2.1 Wound was treated with normal saline group (W+NSS)

The wound was treated with 0.9% normal saline (Sigma Chemical Co, USA) directly once a day until the end of experiment.

3.2.2 Wound implanted with collagen scaffold (W+Coll)

Collagen scaffold was grafted on the wound and fixed with multiple single stitches with a 6-0 nylon suture (Kremer et al, 2000). The wound were not received any treatment until the end of experiment.

3.2.3 Wound treated with 0.03 g/kg.bw *Acanthus ebracteaeus* Vahl. extract (W+AE 0.03)

The wounded mice were treated with W+AE 0.03 directly once a day until the end of experiment.

3.2.4 Wound treated with 0.3 g/kg.bw *Acanthus ebracteaeus* Vahl. extract (W+AE0.3)

The wounded mice were treated with W+AE0.3 directly once a day until the end of experiment.

3.2.5 Wound treated with 3 g/kg.bw *Acanthus ebracteaeus* Vahl. extract (W+AE3)

The wounded mice were treated with W+AE3 directly once a day until the end of experiment.

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3.2.6 Wound implanted with collagen scaffold containing 0.03 g/kg.bw. *Acanthus ebracteaeus* Vahl. extract (W+Coll + AE 0.03)

After the collagen scaffold was grafted on the wound and fixed with multiple single stitches with a 6-0 nylon suture until the end of the experiment, the wound was treated with 0.03 g/kg.bw *Acanthus ebracteaeus* Vahl. directly once a day until the end of experiment.

3.2.7 Wound implanted with collagen scaffold containing 0.3 g/kg.bw. *Acanthus ebracteaeus* Vahl. extract (W+Coll+AE 0.3)

The wound were performed the same as 3.2.6 group but the wound were treated with 0.3 g/kg.bw *Acanthus ebracteaeus* Vahl. extract directly once a day until the end of experiment.

3.2.8 Wound implanted with collagen scaffold containing 3 g/kg.bw. *Acanthus ebracteaeus* Vahl. extract (W+Coll + AE 3)

The wound were performed the same as 3.2.6 group but the wound were treated with 3 g/kg.bw *Acanthus ebracteaeus* Vahl. extract directly once a day until the end of experiment.

Balb/c mice model   22-25g

Sham. N=5

Wound 1.5×1.5 cm ^{2}

| N=15 | N=15 | N=45 | N=45 |

Wound+Veh.

Wound+Collagen
Scaffold

Wound+Acanthus
(0.03, 0.3, 3 g/kg.bw)

Wound+Acanthus+Collagen
Scaffold
(0.03, 0.3, 3 g/kg.bw)

3 days

- Wound area
- Number of neutrophils
  infiltration

7 days

- Wound area
- Capillary vascularity
- Re-epithelization
- Tissue VEGF

14 days

- Wound area
- Capillary vascularity
- Re-epithelization
- Tissue VEGF
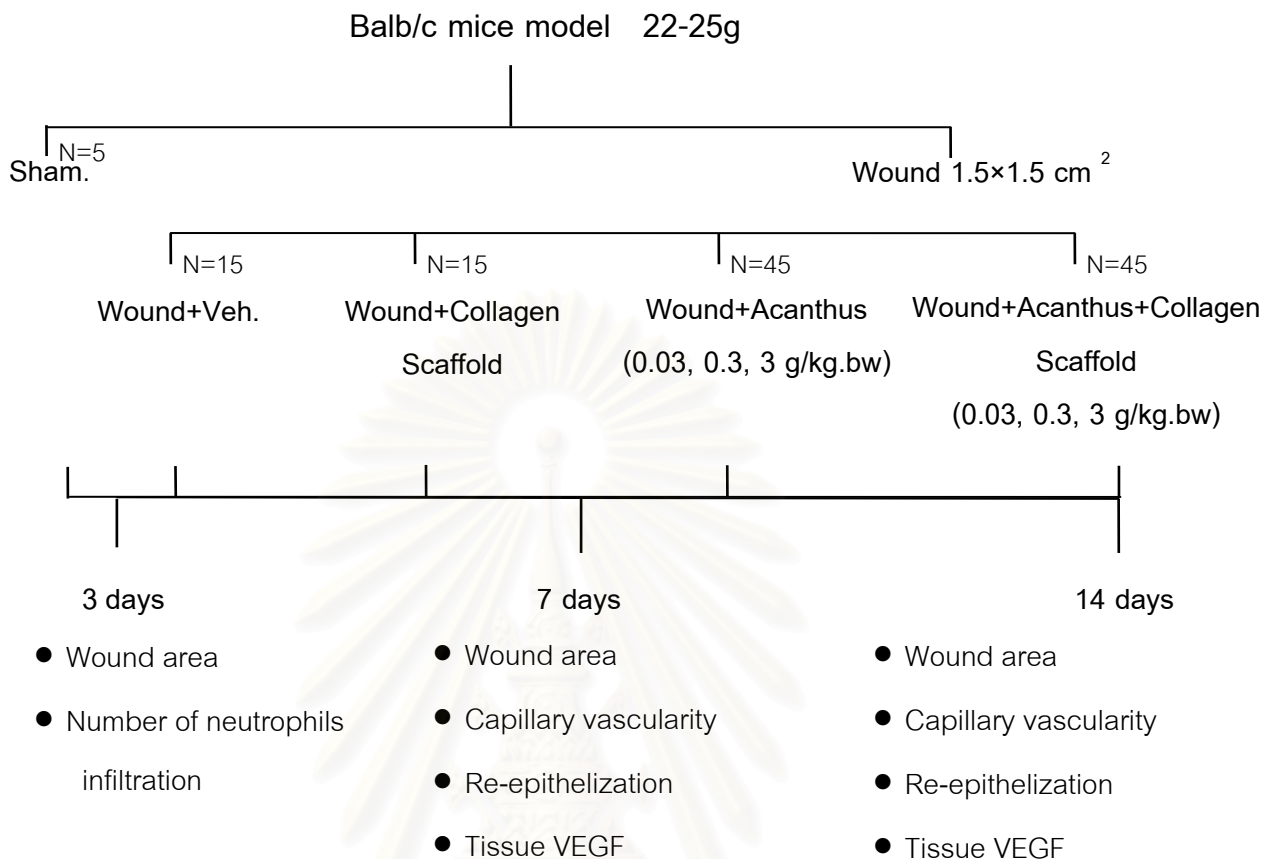
3.3 Wound creation and implantation

After animal was anesthetized by thiopental (pentobarbital) 20µg/ml intraperitoneally, the animal was shaved hair and disinfected with a non iodine-containing alcoholic. Full-thickness wound 1.5×1.5 cm^2 down to the muscle fascia resecting the panniculus carnosus was developed by scissors as shown in Figure 3.1, this wound area was about 10% of total body surface area. Then bovine collagen scaffold was grafted on the wound fixed with multiple single stitches with a 6-0 nylon suture (Michael et al. 2000) as shown in Figure 3.1B.

A   B
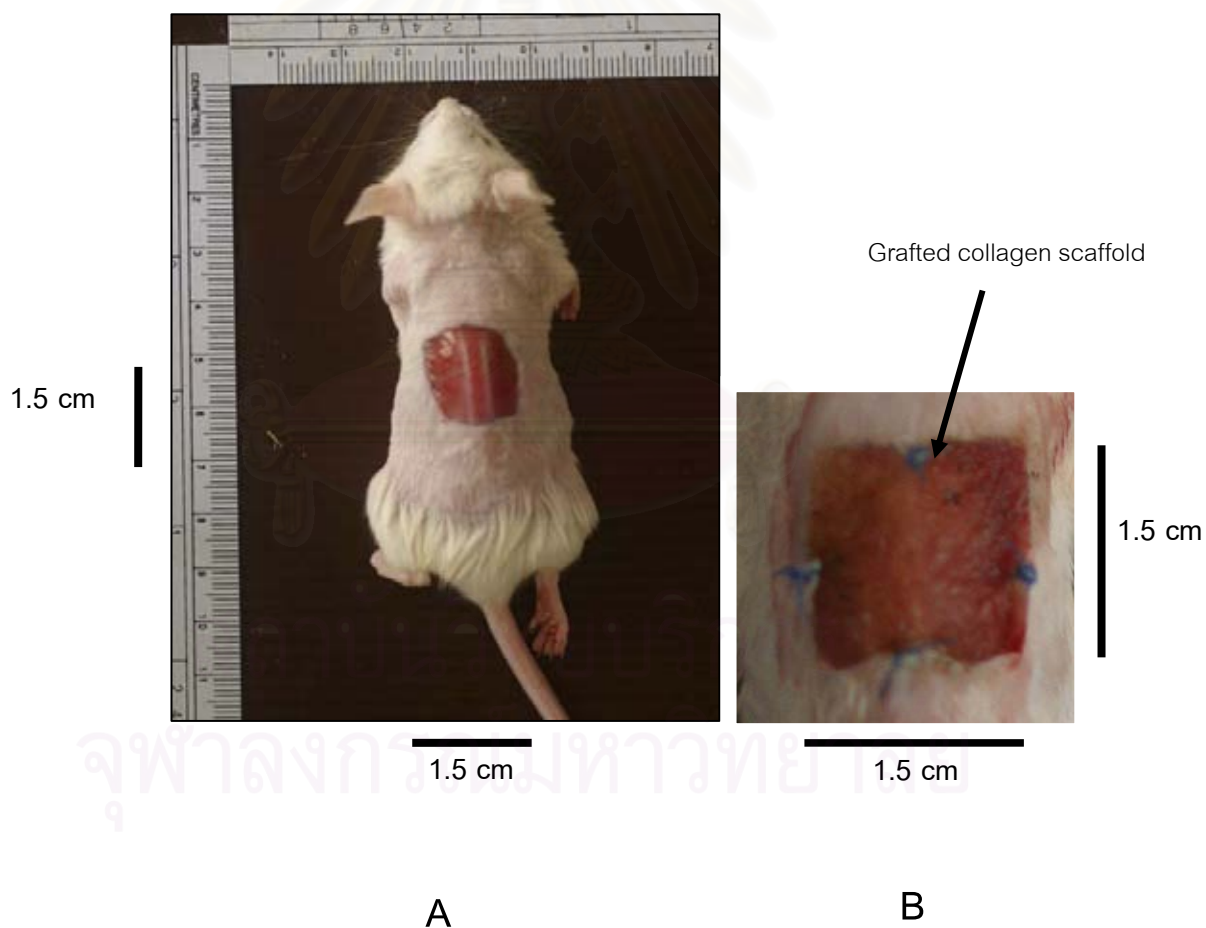
Figure 3.1 Wound created 1.5x1.5 cm^2 (A); Wound was implanted collagen sheet and suture by 6-0 nylon. (B)

Preparation of *Acanthus ebracteatus* Vahl. Extract

The ground stem (5.0 kg) of *Acanthus ebracteatus* was extracted with 95% ethanol (15.0 L) three times. The combined extract was filtered through filter paper. The filtrate was evaporated under reduced pressure at 37 °C to remove ethanol. The concentrated extract was subsequently frozen and freeze-dried to yield 430 g of brown solid mass. These crude extracts (Figure 3.2A) were conducted by Dr. Sanya Hokputsa (Government Pharmaceutical Organization, Bangkok, Thailand). There are different concentrations of the plant extract were prepared to get the treatment doses of 0.03, 0.3, 3 g/kg.bw (Piyaviriyakul et al. 2001). The solution was brown color as shown in Figure 3.2B and used to apply topically on wound area everyday (Lim et al. 2000).
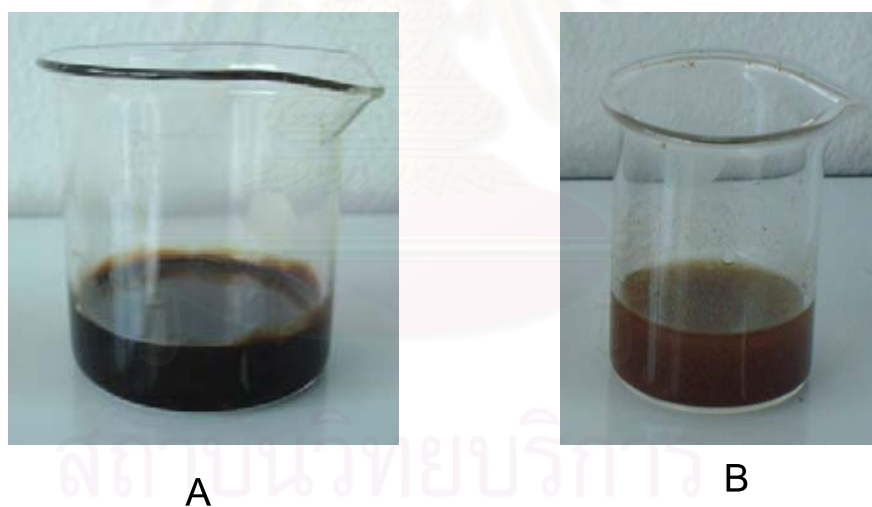
Figure 3.2 The crude extract of *Acanthus ebracteatus* Vahl. (A); *Acanthus ebracteatus* Vahl. in normal saline at a concentration of 0.3 g/kg.bw. (B)

Preparation of bovine collagen scaffold implanted on the wound

The bovine collagen type I (Sigma Chemical Co, USA) was dissolved by acetic acid 0.5 Molar and homogenized by the IKA® T25 digital ULTRA- TURRAX® homogenizer. The homogenate were mold by mental square block, then freeze homogenous block at 4°C temperature. The bovine collagen sheet was then pushed out from the block under the water, and brought to freeze dry and cross link. All these protocols were conducted by i-Tissue Laboratory, Faculty of Medicine, Chulalongkorn University. On the experimental day, the collagen sheet was cut into 1.5×1.5 cm^2 piece and steriled in phosphate buffer saline (PBS) 15 min 3 times before use.
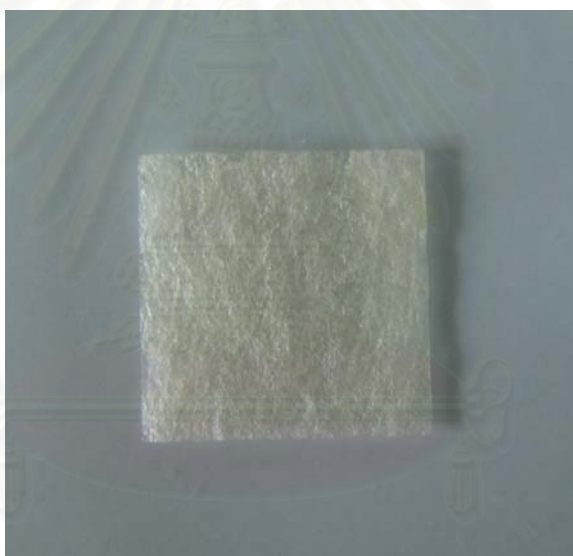
Figure 3.3 1.5×1.5 cm^2 Collagen scaffold produced by i-Tissue Laboratory, Faculty of Medicine, Chulalongkorn University.

D. Experimental protocol

4.1 Measurement of wound closure

Wound closure was defined by decreases in the wound area from day 0 to 3, 7 and 14 of the experiment. The percentage of wound closure was calculated using the following formula:

$$\% \text{ Wound closure} = \frac{\text{Wound area day (0)} - \text{Wound area day (i)}}{\text{Wound area day (0)}} \times 100 \quad \ldots\ldots \ldots\ldots \text{ (1)}$$

To measure the wound area, the wound tissue of each mouse was taken photograph of, using a digital camera (Sony DSC-F828). The wound boundary was traced, and the wound area was analyzed using software (Image-Pro Plus 6.1). Figure 3.4 shows the procedure of measurement of the wound area using the software.
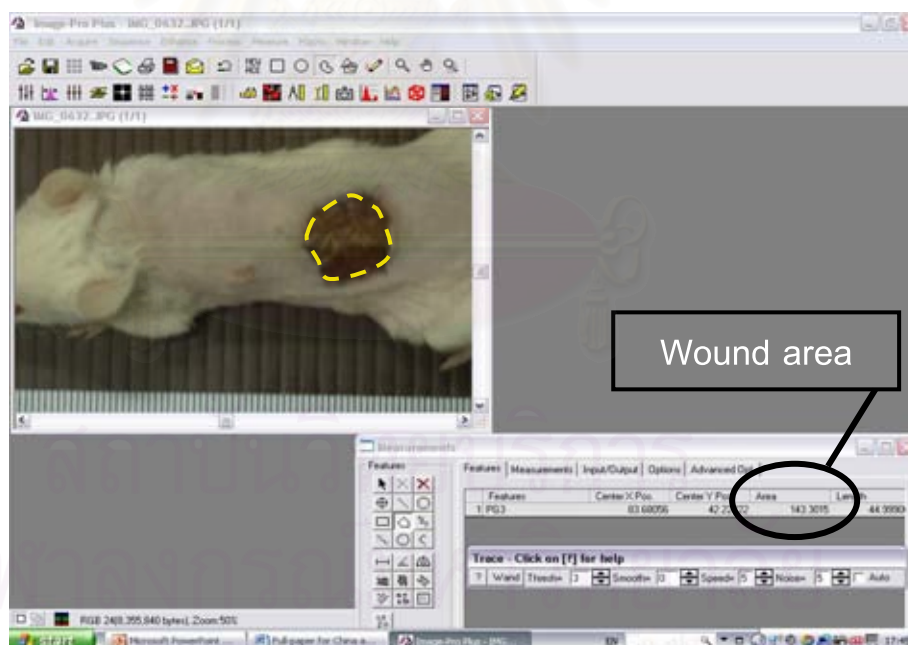
Figure 3.4 Procedure of the wound area (mm^2) measured using Image-Pro Plus 6.1 software.

4.2 Re-epithelialization

A piece of skin at the middle area of wound were fixed in 4% phosphate-buffered formalin for 2–3 days and then embedded in paraffin. Center of paraffin embedded tissue-blocks was serially cut into 5μm thick sections. These sections were stained with hematoxylin-eosin (H&E) by the routine histological method used by Duansak (2007). Based on the H&E slides of each group, re-epithelialization was analyzed using a stereo microscope (Nikon SMZ800, Japan). The re-epithelialization was defined in terms of the ratio between the two distances shown as demonstrated in Figure 3.5 (Hall 1991) according to formula below:

$$\% \text{ Re-epithelialization} = \frac{\text{Distance between of re-epithelialization}}{\text{Distance of the wound edges}} \times 100(\%) \quad \ldots\ldots\ldots\ldots \text{ (2)}$$

Figure 3.5 A example of H&E slide of wound used to determine re-epithelialization. Re-epithelialization was calculated as the ratio between the distance covered by the new epithelium (r) and the distance between wound edges (R).

4.3 Measurement of capillary vascularity (CV)

To study the development of angiogenesis during wound healing, changes in capillary vascularity (CV) in wound tissue was examined on day 7, 14. The CV was defined by the ratio of capillary to tissue area. This is often used as an indicator of the capillary vascularity.

To measure the CV level, the animal was anesthetized by intraperitonial injection thiopental (pentobarbital) 20µg/ml intraperitoneally. The dorsal skin around the wound was cut open using scissors. The wound area was cleared using a forceps. Figure 3.6 (A, B) show examples of dorsal skin with an area of wound area.

A   B

Figure 3.6 The open dorsal skin of wound area (A). The arrow is the area of wound (B).

To obtain fluorescence image of the microvasculature of the skin flap, intravital fluorescence video microscopy technique was used. Inserting a polyethylene catheter (PE 10, inner diameter of 0.28 mm.) into the right jugular vein, we injected 0.2 ml of 5% FITC-labeled dextran 150000 (Sigma Chemical Co, USA) for contrast-enhancement by staining of plasma. Figure 3.7 shows the fluorescence video microscopic system used in this experiment. The equipment set consisted of a video microscope (Nikon E50i, Tokyo, Japan) with a 10x objective and eyepieces lens, a camera controller (Hamamatsu C2400, Japan), a video timer (For. A VTG-33, Japan) and a video recorder (Panasonic NV-HD620, Japan). During the intravital experiment, the studied area was kept at moisture with normal saline dropped on the wound flap (2 ml/min). The body

temperature of the animal was control at 36-37°C by a hot pad. The FITC-labeled microvasculature was observed and recorded in real-time using a set of intravital fluorescence microscopic equipment.
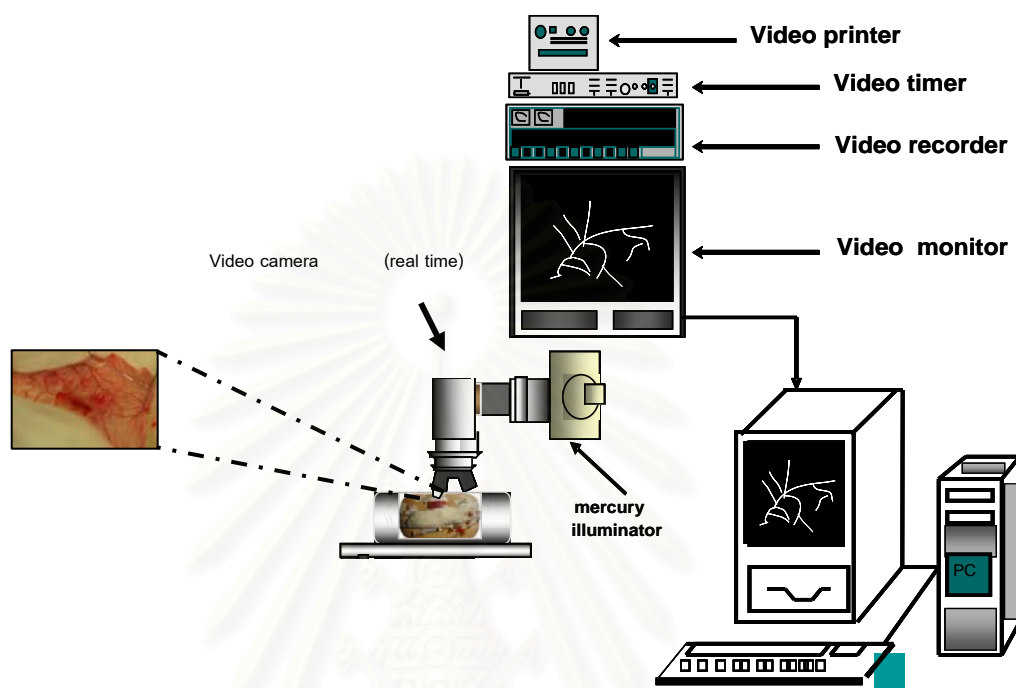
Figure 3.7 Intravital fluorescence video microscopic system used for intravital observation of the microcirculation in the wound area.

Video image of each animal was used to analyze changes in capillary vascularity on day 7 and 14 Figure 3.8 (A, B) shows the procedure of measurement of the CV using the software Image Pro-plus. In the present measurement, we considered capillaries whose diameter was less than 10 µm. The following steps of measurement were used to assess the capillary vascularity:

1) Capillary network were randomly selected and captured.

2) On one frame, three regions of interest (ROIs) were carefully selected by avoiding relatively larger vessels whose diameter are more than 10 µm, so that ROIs might cover the area of neocapillaries only. Each ROI was set to include 100x100 pixels.

3) Number of pixel located within all capillaries in each ROI was calculated using Global Lab II software.

4) The percentage of capillary vascularity was caluculated using the following equation:

% CV = $\dfrac{\text{(Number of pixels within the capillaries) x 100 (\%)}}{\text{(Total numbers of pixels within the 100X100 window frame area)}}$ ………… ….. (3)

Figure 3.8A Picture of capillary from video recorder.
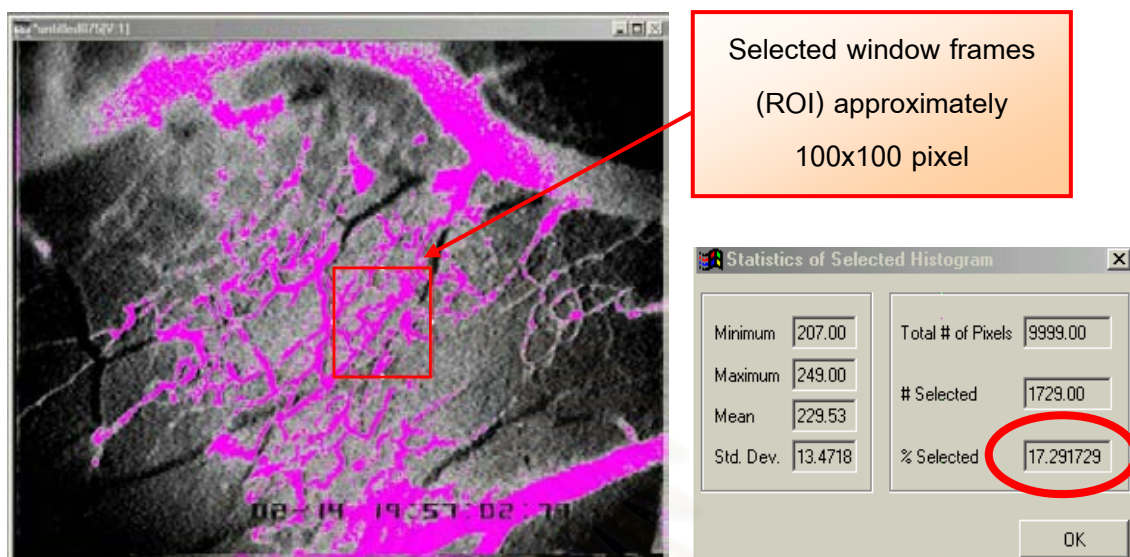
Figure 3.8B To determine capillary vascularity, the selected window (100x100 pixels) of videoimage containing only neocapillaries network was analyzed by using Global Lab Image II

4.4 ELISA analysis of wounded tissue VEGF

To determine wounded tissue VEGF levels, a piece of skin was excised from the dorsal part of mice and frozen at -80 °C. On the day o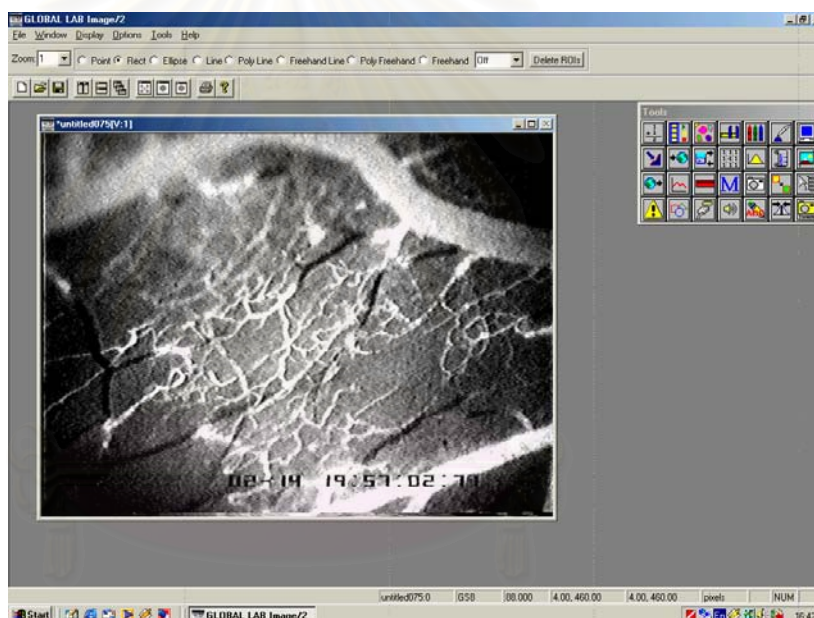f experiment, the piece of tissue was then rinsed with 1X PBS to remove excess blood, homogenized in 20 mL of 1X PBS, and stored overnight at -20° C. After two freeze-thaw cycles were performed to break the cell membranes, the homogenates were centrifuged for 5 minutes at 5000 x g. Samples can be assayed immediately or stored at -20° C. Avoid repeated freeze-thaw cycles. Fifty ml of each sample was be used to determine VEGF levels. Immunoreactive VEGF will be quantified using a sandwich ELISA (Quantikine M Mouse VEGF Immunoassay kit; R & D System, Minneapolis, MN) according to the manufacturer's recommended protocol.

Preparation of mouse VEGF standard

The standard curve was generated by 8 serial concentrations of 0, 7.8, 15.6, 31.2, 62.5, 125, 250, 500 pg/mL mouse VEGF standard. The following recommended by manufacturer was used to make up the kit standard in the serial dilution.

1. Label 7 micro tubes following the concentration.

2. Add 200 µl of Calibrator Diluents RD5T into each tube except highest tube.

3. Add 200 µl of mouse VEGF standard concentration 500 pg/mL into the tube 250 pg/mL and produce a 2-fold dilution series as shown in Figure 3.8

4. Mix each tube thoroughly before the next transfer.
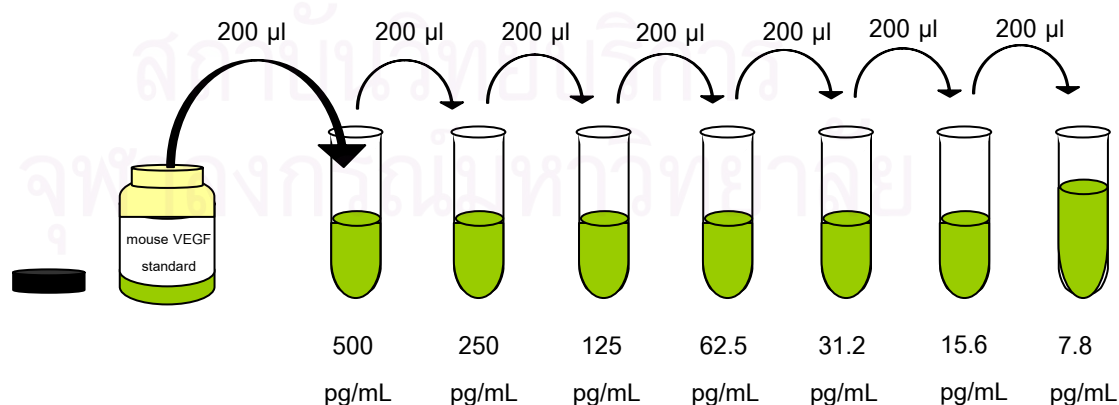
Figure 3.9 Preparation of mouse VEGF standard

The experimental procedures were performing as following:

1. Bring and prepare all reagents to room temperature.

2. Add 50 µl assay diluents to each well.

3. Add 50 µl standard and sample to each well and tap plate for one minute. Cover the plate and incubate for 2 hours at room temperature.

4. Aspirate and wash each well five times.

5. Add 100 µl conjugate to each well. Cover the plate and incubate for 2 hours at room temperature.

6. Aspirate and wash each well five times.

7. Add 100 µl substrate solutions to each well. Incubate 30 minutes at room temperature. Protect from light.

8. Add 100 µl stop solution to each well.

9. Read optical density at 450 nm by using Microplate reader, BIO-RAD Model 680.

Calculation of results

The average of duplicate testing was used for both standard and samples. Standard curve was conducted by using excel chart wizard tool. The calibration graph and the equation of best fitted linear line are show in Figure 3.10

VEGF Std (pg/ml)	Optical density			
	I	II	AV	Collected
0	0.15	0.16	0.16	0.00
7.8	0.30	0.22	0.26	0.10
15.6	0.21	0.20	0.21	0.05
31.2	0.35	0.34	0.35	0.19
62.5	0.69	0.64	0.66	0.51
125	1.21	1.28	1.24	1.09
250	2.11	1.91	2.01	1.85
500	3.18	2.85	3.02	2.86
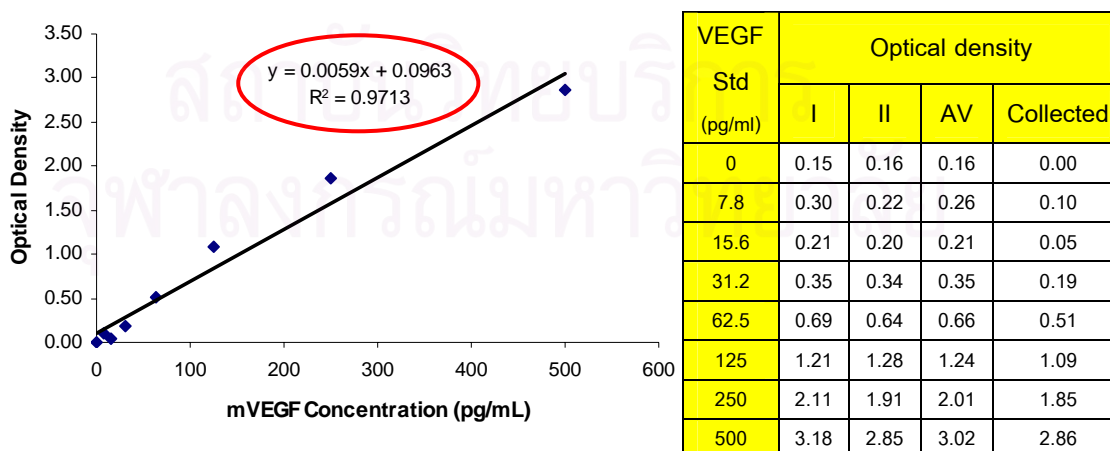
Figure 3.10 Calculation of optical density

4.5 Measurement of the number of neutrophil infiltration

Skin from around of wound area was fixed in 4% phosphate-buffered formalin for 2–3 days and then embedded in paraffin. From the paraffin embedded tissue-blocks, edges of wound tissue (5µm) sections were serially cut and stained with hematoxylin-eosin (H&E). All histopathology examinations were performed by the same pathologist with 2 blind tests. The specimens were evaluated for inflammatory reaction. Under X40 magnification, 0.25 mm^2 were examined by using microscope Eclipse E600 and their mean was recoded by digital camera DXM1200F (Sakallıoglu et al. 2004). Picture was analyzed by tool in Image Pro Plus software.

CHAPTER IV

RESULTS

This chapter is composed of five parts of results obtained using the mouse model of full-thickness wound. These were used to determine the effects of AE with collagen scaffold on the angiogenesis and wound closure.

A. Effect of AE with collagen scaffold on the neutrophil infiltration

Table 4.1 and Figure 4.1 show number of neutrophils infiltration (means ± SEM) in five groups. Number of neutrophils in W+NSS group significantly increased more than that in sham group. The W+AE-treated groups (doses 0.03, 0.3 g/kg.bw) showed no significant differences, compared to W+NSS. The W+Coll group showed the higher number of neutrophils infiltration significantly, compared to W+NSS (p=0.03). W+Coll+AE0.03 and W+Coll+AE0.3 groups showed no significant difference, compared to W+NSS, but combination of W+Coll+AE0.3 reduced number of neutrophil significantly, compare to W+Coll group.

B. Effect of AE with collagen scaffold on the wound closure

Tables 4.2-4.4 and Figures 4.2-4.4 show percentage of wound closure (means ± SEM) on days 3, 7 and 14.

On day 3, W+AE0.03 showed that the percentage of wound closure in W+AE0.03 no significant difference, compared to W+NSS group, but it was significantly higher than W+AE0.3 (p=0.04). The W+Coll, W+Coll+AE0.03 and W+Coll+AE0.3 group, there were not different significantly, (Table 4.2 and Figure 4.2).

On day 7, the percentage of wound closure in W+AE group was not significantly different, compared to that in W+NSS group. The W+Coll group was significantly higher than W+NSS group, which wound W+Coll+AE0.3 significantly increased, compared to W+NSS group (p=0.01, p=0.03, respectively). However, there

was no significant difference between groups of W+Coll, and both W+Coll+AE (Table 4.3 and Figure 4.3).

On day 14, the W+AE treated groups (0.3, 0.03) were significantly increased compared to W+NSS group (p=0.004 and p=0.01 respectively). In addition, the W+Coll, W+Coll+AE0.03 and W+Coll+AE0.3 significantly increased more than W+NSS group (p=0.01, p=0.02 and p=0.02 respectively), (Table 4.4 and Figure 4.4).

C. Effect of AE with collagen scaffold on re-epithelialization

The re-epithelialization was used to evaluate wound healing on day 7 and 14 after operation.

On day 7, Table 4.5 and Figure 4.5 shows means ± SEM of %re-epithelialization, showed no significant difference. However, on day 14, the increase in re-epithelialization was observed in AE groups, compared to W+NSS. In particular, the W+AE 0.03 showed %re-epithelialization of significant increase compared to W+NSS group (p=0.05). The %re-epithelialization of W+Coll and W+Coll+AE0.3 groups were increased significantly compare to W+ NSS group (p=0.05) (Table 4.6 and Figure 4.6).

D. Effect AE with collagen scaffold on the angiogenesis in the wound area

The angiogenesis during wound healing was evaluated in terms of changes in the capillary vascularities increased on day 7 and 14. Table 4.7-4.8 and Figure 4.7-4.8 show mean values and standard deviation of mean.

On day 7, %CV of W+AE0.03 and W+Coll+AE0.3 groups were significantly increased, compared to W+NSS group (p=0.001, p=0.03 respectively). The %CV of W+Coll+AE0.3 was significantly higher than that in W+NSS group (p=0.03) (Table 4.7 and Figure 4.7).

On day 14, %CV in W+Coll+AE0.3 was significantly higher than that in W+NSS (p=0.002). In addition, W+Coll+AE0.3 group showed significantly higher than CV level W+Coll+AE0.03 group (p=0.05) (Table 4.8 and Figure 4.8).

E. Effect of W+AE with collagen scaffold on the tissue growth VEGF level

The VEGF level was measured in the collected tissue located in the wound area (see Methodology). Table 4.9-4.10 and Figure 4.9-4.10 show number of VEGF levels (means ± SEM) determined using ELISA kit.

On day 7, VEGF levels of all groups showed no significant difference, except, W+Coll+AE0.3 group, compared to W+Coll group (p=0.001).

On day 14, the results showed no significant difference among all groups.

Table 4.1 Means ± SEM of number of neutrophil infiltration /0.25mm^2 on day 3 in Sham, Wound with vehicle (W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+AE0.03), *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+AE0.3), Collagen scaffold (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+Coll+AE0.3)

GROUP	Numbers of neutrophil/0.25mm^2	P-value
W+NSS	68.0 ± 9.8 (n=5)	0.03
W+AE0.03	70.8 ± 5.4 (n=5)	0.03
W+AE0.3	102.4± 27.4 (n=5)	0.03
W+Coll	145.8±6.5 (n=5)	0.02
W+Coll+AE0.03	110.0±29.8 (n=5)	0.02
W+Coll+AE0.3	61.0±9.2 (n=5)	0.02

The number of neutrophil infiltration/0.25mm^2 on day 3

Figure 4.1 Graphs showing the means ± SEM of the numbers of neutrophil infiltration/0.25mm^2 on day 3

Table 4.2 Means ± SEM of percent of wound closure on day 3 in Wound with vehicle
(W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+AE0.03), *Acanthus ebracteatus*
Vahl. 0.3 g/kg.bw (W+AE0.3), Collagen scaffold (W+Coll), Collagen scaffold
combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and
Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw
(W+Coll+AE0.3)

GROUP	% Wound closure 3 Days	P-value
W+NSS	30.7±3.6 (n=5)	
W+AE 0.03	31.4±4.7 (n=5)	0.04 } 0.05
W+AE 0.3	19.8±3.8 (n=5)	
W+Coll	35.5±6.0 (n=4)	
W+Coll+AE0.03	26.9±3.3 (n=5)	
W+Coll+AE0.3	29.1±0.7 (n=5)	

Figure 4.2 Graphs showing the means ± SEM of percent of wound closure on day 3

Table 4.3 Means ± SEM of percent of wound closure on day 7 in Wound with vehicle (W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+AE0.03), *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+AE0.3), Collagen scaffold (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+Coll+AE0.3)

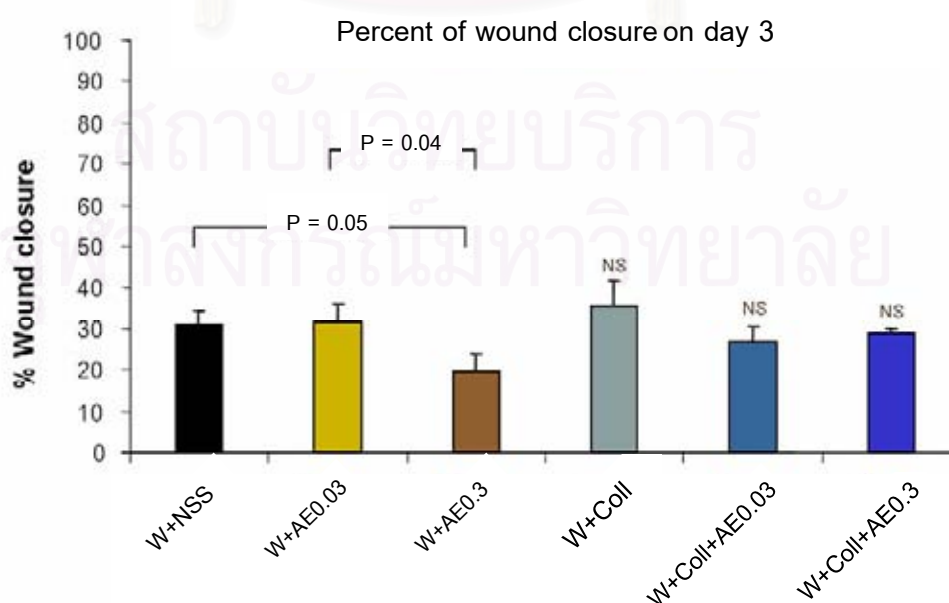| GROUP	% Wound closure	P-value
	7 Days	
W+NSS	47.0±3.2	
	(n=5)	
W+AE 0.03	50.3±1.0	
	(n=5)	0.01
W+AE 0.3	36.6±5.6	
	(n=5)	0.03
W+Coll	59.1±2.1	
	(n=4)	
W+Coll+AE0.03	54.2±3.1	
	(n=5)	
W+Coll+AE0.3	57.9±0.6	
	(n=5)	

Figure 4.3 Graphs showing the means ± SEM of percent of wound closure on day 7

Table 4.4 Means ± SEM of percent of wound closure on day 14 in Wound with vehicle (W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+AE0.03), *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+AE0.3), Collagen scaffold (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+Coll+AE0.3)

GROUP	% Wound closure 14 Days	P-value
W+NSS	90.8±3.7 (n=5)	0.004
W+AE 0.03	98.4±0.7 (n=5)	0.01
W+AE 0.3	97.9±0.9 (n=5)	0.01
W+Coll	97.5±1.1 (n=5)	0.02
W+Coll+AE0.03	96.6±0.6 (n=5)	0.002
W+Coll+AE0.3	98.9±0.5 (n=5)	

Figure 4.4 Graphs showing the means ± SEM of percent of wound closure on day 14

Table 4.5 Means ± SEM of re-epithelialization on day 7 in Wound with vehicle (W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+AE0.03), *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+AE0.3), Collagen scaffold (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+Coll+AE0.3)

GROUP	Re-epithelialization	P-value
	7 Days	
W+NSS	52.5±6.7	
	(n=4)	
W+AE 0.03	53.8±12.0	
	(n=4)	
W+AE 0.3	36.0±7.2	
	(n=4)	
W+Coll	47.6±14.0	
	(n=3)	
W+Coll+AE0.03	61.3±10.9	
	(n=3)	
W+Coll+AE0.3	56.7±10.6	
	(n=5)	
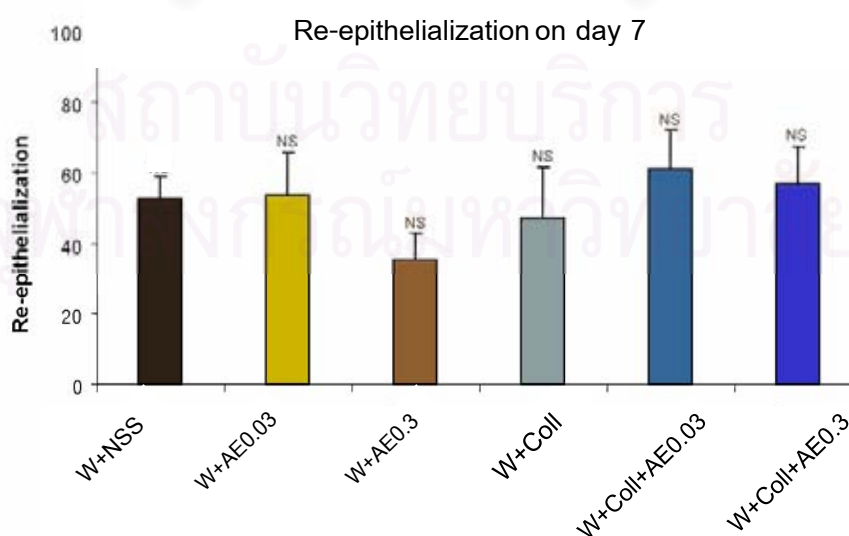
Figure 4.5 Graphs showing the means ± SEM of re-epithelialization on day 7

Table 4.6 Means ± SEM of re-epithelialization on day 14 in Wound with vehicle (W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw, (W+AE0.03), *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw, (W+AE0.3), Collagen scaffold (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+Coll+AE0.3)

| GROUP	Re-epithelialization 14 Days	P-value
W+NSS	89.4±5.1 (n=5)	0.05
W+AE 0.03	100.00±0 (n=5)	0.05
W+AE 0.3	96.1±2.5 (n=5)	0.05
W+Coll	100.0±0 (n=5)	
W+Coll+AE0.03	98.5±1.5 (n=5)	
W+Coll+AE0.3	100.0±0 (n=5)	

Figure 4.6 Graphs showing the means ± SEM of re-epithelialization on day 14

Table 4.7 Means ± SEM of angiogenesis on day 7 in Wound with vehicle (W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+AE0.03), *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+AE0.3), Collagen scaffold (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+Coll+AE0.3)

GROUP	Capillary vascularity (%)	P-value
	7 Days	
W+NSS	10.7±0.3	
	(n=5)	0.001
W+AE 0.03	18.7±0.7	
	(n=4)	
W+AE 0.3	15.9±1.6	
	(n=5)	
W+Coll	14.9±2.3	0.03
	(n=4)	
W+Coll+AE0.03	14.7±2.2	
	(n=5)	
W+Coll+AE0.3	15.5±1.4	
	(n=5)	

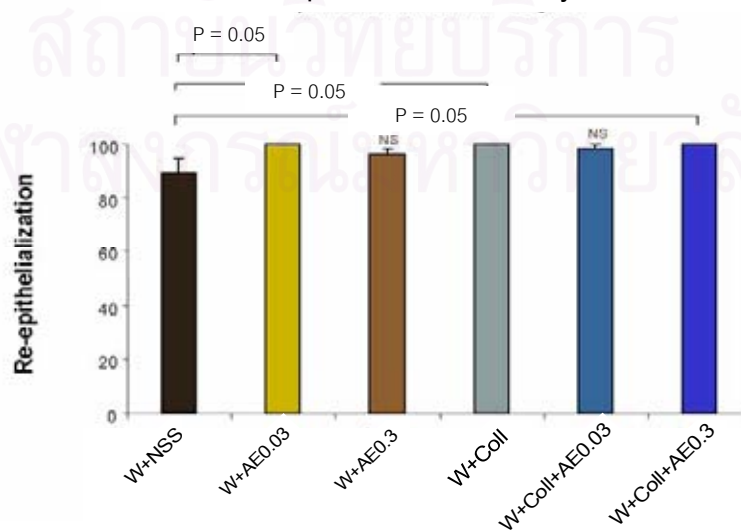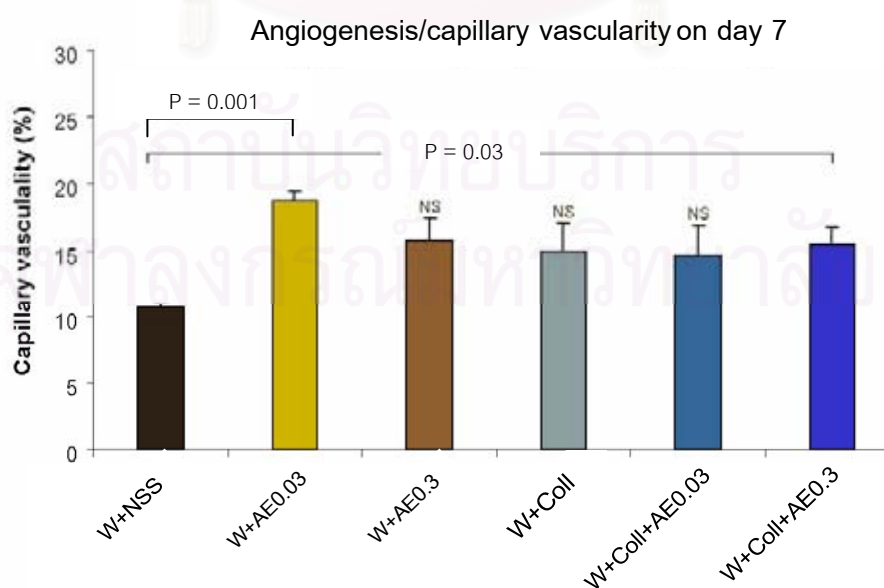
Figure 4.7 Graphs showing the means ± SEM of angiogenesis on day 7

Table 4.8 Means ± SEM of angiogenesis on day 14 in Wound with vehicle (W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+AE0.03), *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+AE0.3), Collagen scaffold (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+Coll+AE0.3)

GROUP	Capillary vascularity (%)	P-value
	14 Days	
W+NSS	9.0±0.8	
	(n=5)	
W+AE 0.03	15.2±4.6	
	(n=5)	
W+AE 0.3	15.2±4.6	
	(n=5)	0.05
W+Coll	17±3.5	
	(n=4)	
W+Coll+AE0.03	13.1±0.5	
	(n=3)	0.002
W+Coll+AE0.3	23.1±2.9	
	(n=5)	

Angiogenesis/capillary vascularity on day 14
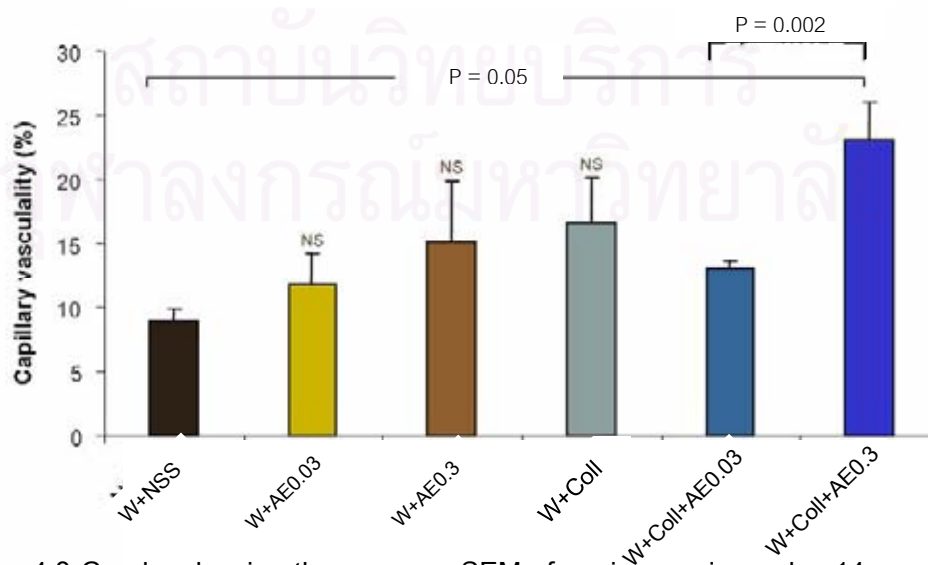
Figure 4.8 Graphs showing the means ± SEM of angiogenesis on day 14

Table 4.9 Means ± SEM of the level of VEGF in the skin tissue on day 7 in Wound with vehicle (W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+AE0.03), *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+AE0.3), Collagen scaffold (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+Coll+AE0.3)

GROUP	VEGF concentration (pg/ml)	P-value
	7 Days	
W+NSS	48.3±6.1	
	(n=4)	
W+AE 0.03	50.9±1.5	
	(n=5)	
W+AE 0.3	49.7±7.0	
	(n=5)	
W+Coll	34.5±7.2	
	(n=5)	
W+Coll+AE0.03	37.1±5.1	0.01
	(n=5)	
W+Coll+AE0.3	63.8±3.3	
	(n=5)	

The tissue VEGF level on day 7
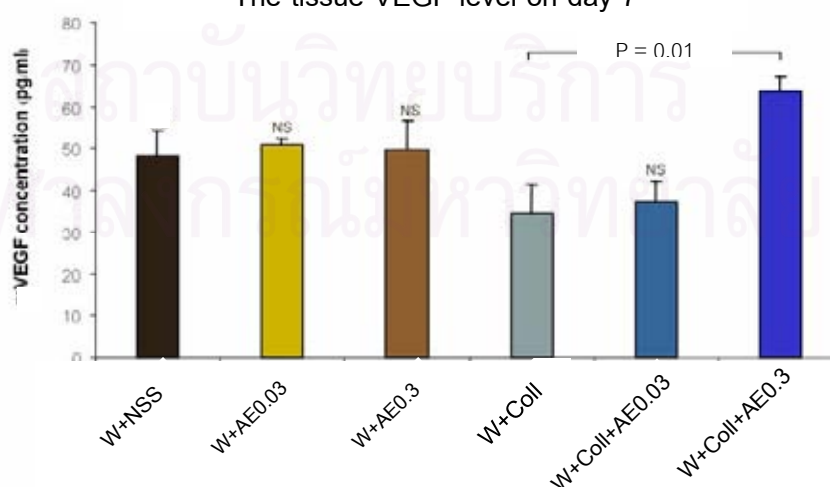
Figure 4.9 Graphs showing the means ± SEM of the level of VEGF in the skin tissue on day 7

Table 4.10 Means ± SEM of the level of VEGF in the skin tissue on day 14 in the group of Wound with vehicle (W+NSS), *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw, (W+AE0.3), *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw, Collagen scaffold (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.03 g/kg.bw (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw (W+Coll+AE0.3)

GROUP	VEGF concentration (pg/ml)	P-value
	14 Days	
W+NSS	22.3±2.7	
	(n=4)	
W+AE 0.03	35.5±12.2	
	(n=5)	
W+AE 0.3	17.8±2.6	
	(n=4)	
W+Coll	34.9±8.2	
	(n=4)	
W+Coll+AE0.03	39.1±9.2	
	(n=4)	
W+Coll+AE0.3	28.0±4.2	
	(n=4)	
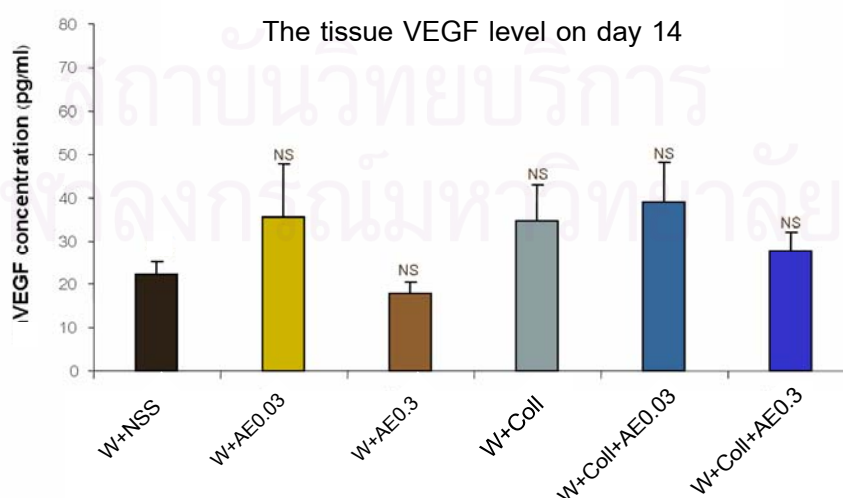
Figure 4.10 Graphs showing the means ± SEM of the level of VEGF in the skin tissue on day 14

Figure 4.11 Neutrophil infiltration of Sham and full-thickness wound topical with NSS (W+NSS), *Acanthus ebracteatus* Vahl. dose 0.03 (W+AE0.03), *Acanthus ebracteatus* Vahl. dose 0.3 (W+AE0.3), Collagen scaffold implanted (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. dose 0.03 (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. dose 0.3 (W+Coll+AE0.3) on day 3 after wound operation.

W+NSS W+AE0.03

W+AE0.3 W+Coll

W+Coll+AE0.03 W+Coll+AE0.3
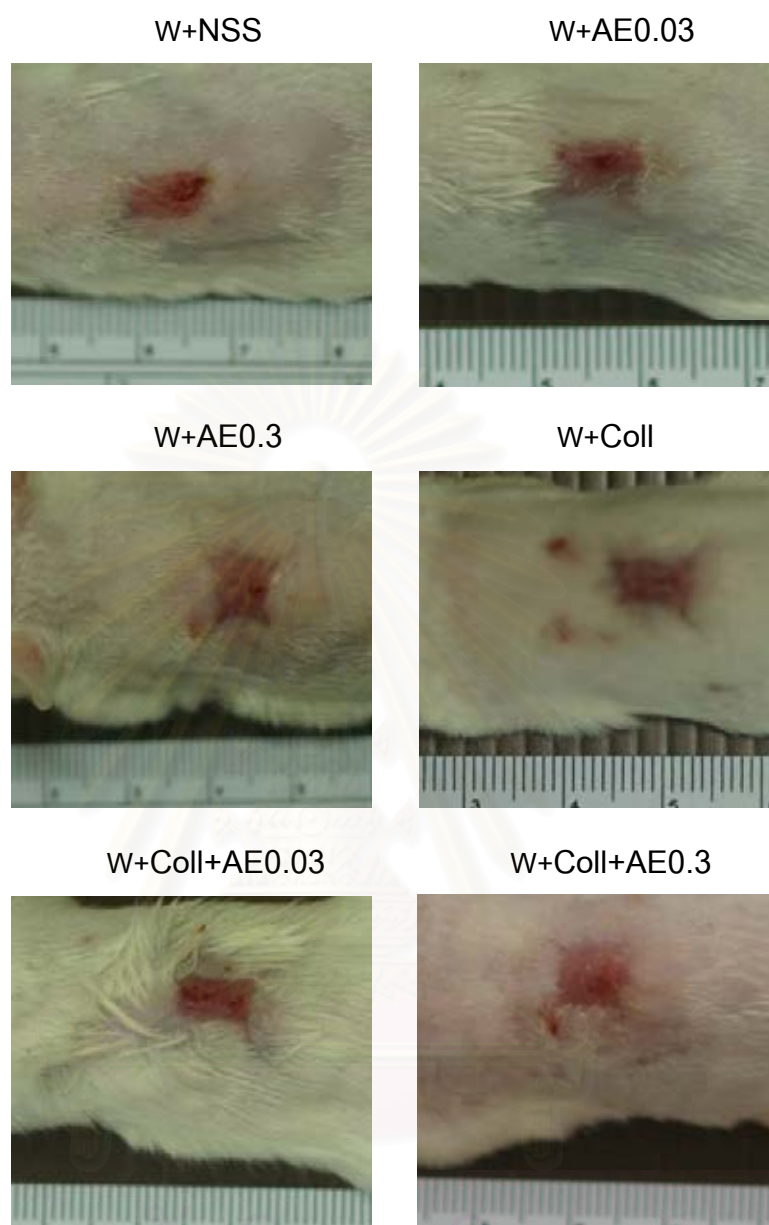
Figure 4.12 Wound area of full-thickness wound topical with NSS (W+NSS), *Acanthus ebracteatus* Vahl. dose 0.03 (W+AE0.03), *Acanthus ebracteatus* Vahl. dose 0.3 (W+AE0.3), Collagen scaffold implanted (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. dose 0.03 (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. dose 0.3 (W+Coll+AE0.3) on day14 after wound operation.

W+NSS

W+AE0.03

W+AE0.3

W+Coll

W+Coll+AE0.03
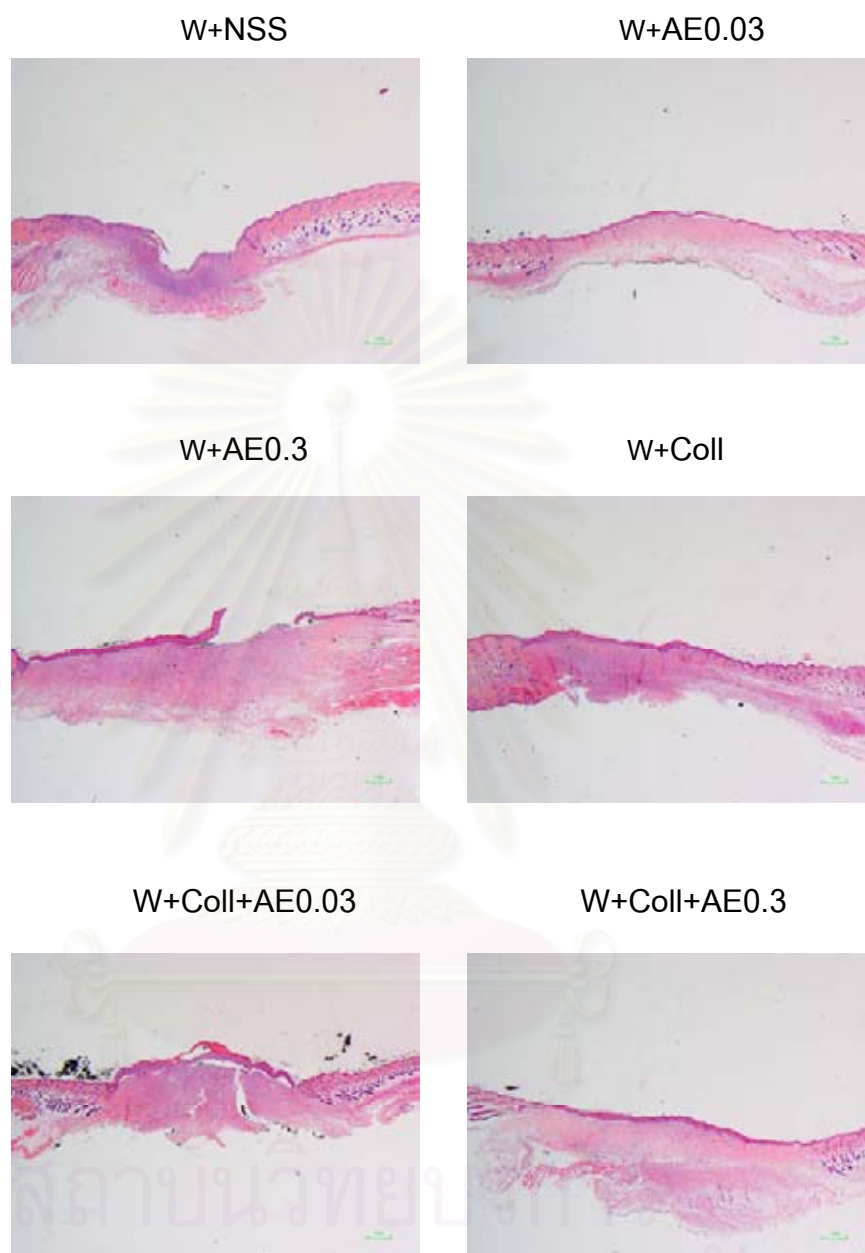
W+Coll+AE0.3

Figure 4.13 Re-epithelialization of full-thickness wound topical with NSS (W+NSS), *Acanthus ebracteatus* Vahl. dose 0.03 (W+AE0.03), *Acanthus ebracteatus* Vahl. dose 0.3 (W+AE0.3), Collagen scaffold implanted (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. dose 0.03 (W+Coll+AE0.03) and   Collagen scaffold combination with *Acanthus ebracteatus* Vahl. dose 0.3 (W+Coll+AE0.3) on day14 after wound operation.
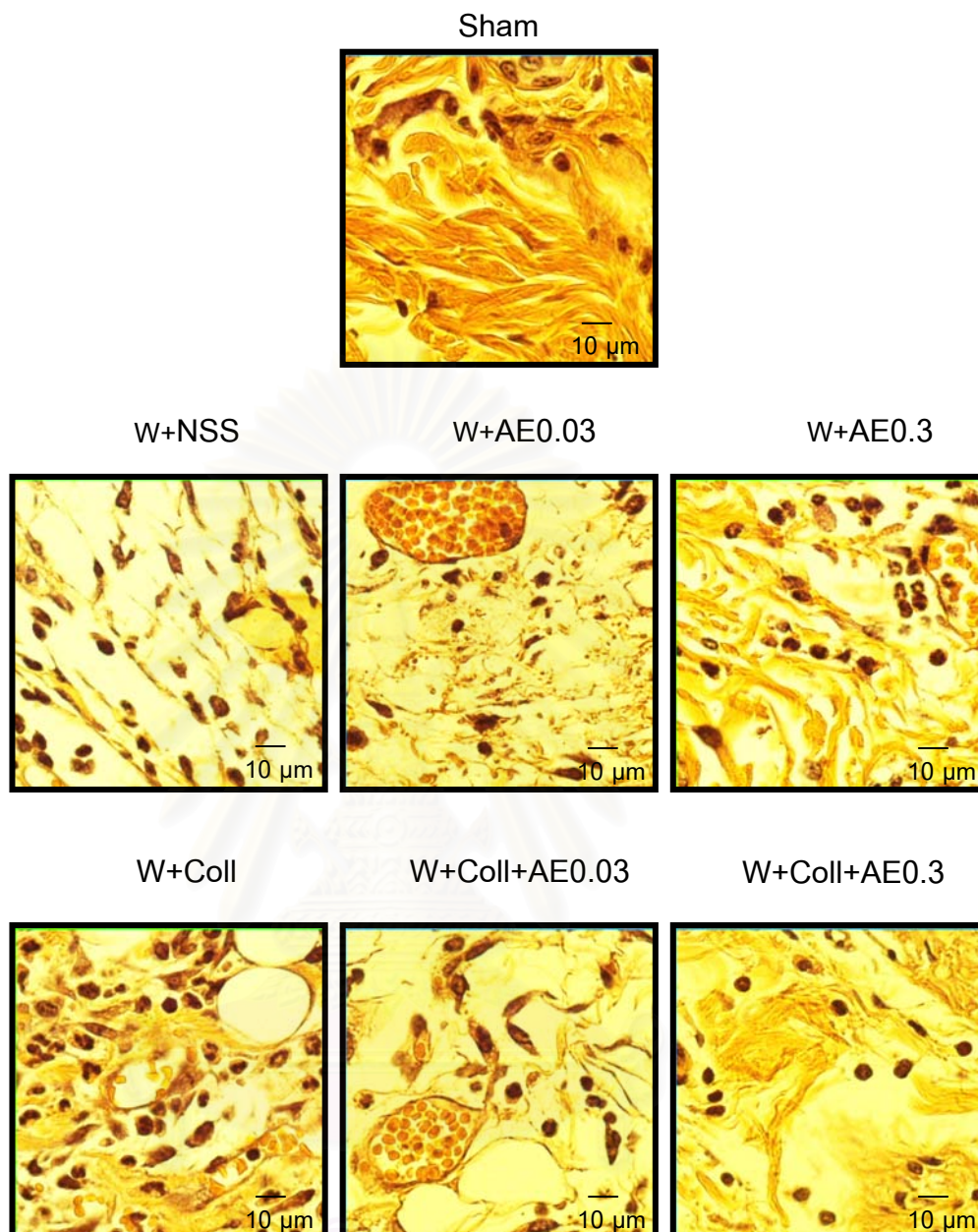
W+NSS

W+AE0.03

W+AE0.3

W+Coll

W+Coll+AE0.03

W+oll+AE0.3
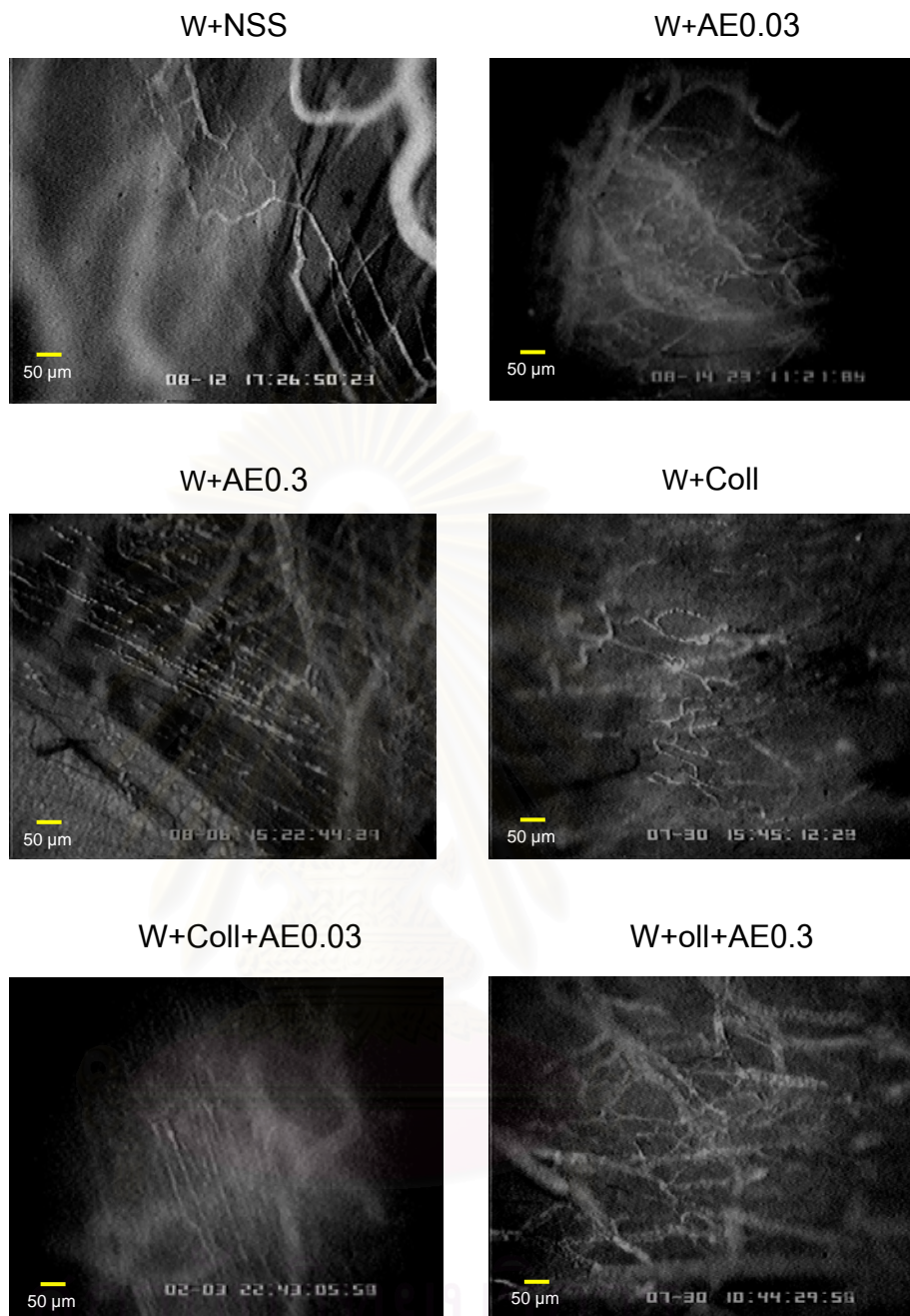
Figure 4.14 Angiogenesis of full-thickness wound topical with NSS (W+NSS), *Acanthus ebracteatus* Vahl. dose 0.03 (W+AE0.03), *Acanthus ebracteatus* Vahl. dose 0.3 (W+AE0.3), Collagen scaffold implanted (W+Coll), Collagen scaffold combination with *Acanthus ebracteatus* Vahl. dose 0.03 (W+Coll+AE0.03) and Collagen scaffold combination with *Acanthus ebracteatus* Vahl. dose 0.3 (W+Coll+AE0.3) on day14 after wound operation.

CHAPTER V

DISCUSSION

The present experiment was conducted to evaluate the effects of AE extract combined with bovine collagen scaffold on angiogenesis and wound closure in the full-thickness wound mice model.

The results were demonstrated in terms of treatment duration (3, 7, 14 days) and doses difference (0.03 and 0.3 g/kg bw daily). The result of experimental trial, using daily topical AE at the highest dose (3 g/kg bw), could not be obtained, because the solution was too sticky, so it could not diffuse through the collagen scaffold. Furthermore, it also caused the tissue to be infected as shown in Figure 5.1.

For these reasons, the researcher has decided not to use the highest dose of AE in this study.
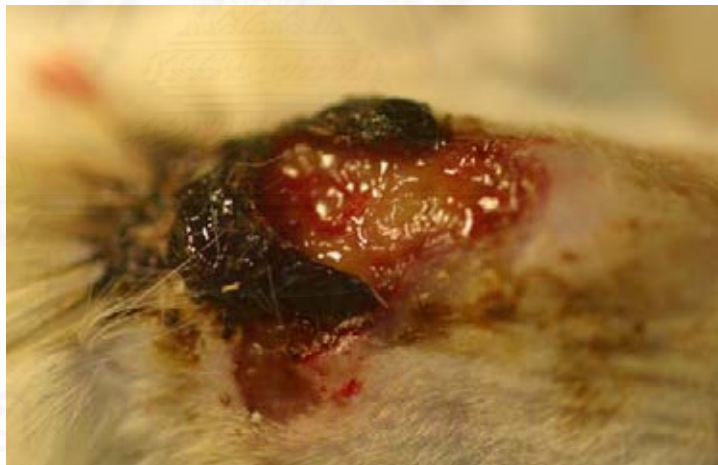
Figure 5.1. The 14th-day wound daily treated with 3mg/kg bw of AE (W+AE3 group) was shown more severe inflammation.

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A. The effect on the number of neutrophils infiltration

As reported by Goldsmith, LA. (1991) inflammation exists in the early phase of wound healing, 3-4 days after wound injury. We performed the experiment to assess the effect of AE with collagen scaffold on this early inflammatory phase by using H&E staining. The number of neutrophils infiltration on day 3 was evaluated using Software (see the methodology). The numbers of neutrophils infiltration significantly increased in wounded mice, compared to sham group (Table 4.1).

Bovine collagen scaffold is a foreign matter to the living tissue, so that the wound be riddled with neutrophils. In bovine collagen-scaffold implanted skin, neutrophils infiltrated increased in the number. Interestingly, W+AE (0.3g/kg bw) in combination with bovine collagen scaffolds significantly reduced numbers of neutrophils, compared to W+Coll-group, but their difference was not significant, compared to the W+NSS group (Table 4.1 and Figure 4.1). These indicated that W+AE0.3 could be used as an anti-neutrophils infiltration induced by the implantation of bovine collagen scaffold for 3-day duration. This effect did not appear clearly for less amount of neutrophils infiltration without bovine collagen scaffold.

Previously, Kongcharoensuntorn et al. (2005) showed that there were three kinds of bioactive compounds in AE, (Flavonoids, Phenylpropanoids and β-sitosterol) which could act as anti-inflammatory agents. These active compounds in AE might be ones that could reduce bovine-collagen-scaffold-induced acute inflammation.

B. The effect on the wound closure

In cases of severe and large amounts of skin loss, immediate coverage of the wound surface with bovine collagen scaffold were used for protecting the area from the loss of fluid and proteins, preventing infection through bacterial invasion, and subsequent tissue damage. Besides, Freyman TM, et al. (2001), also showed the benefit of collagen scaffold was to enhance skin regeneration and to reduction of scar formation.

In our study by using the model of full-thickness wound, the effect of bovine collagen scaffold on enhancing wound closure was significantly demonstrated on day 7 and day 14 (Tables 4.3-4.4 and Figures 4.3-4.4). By other treatments of W+AE0.03, W+AE0.3, W+Coll+AE0.03 and W+Coll+AE0.3, the significant increase in wound closure were observed on day 14 as compared to W+NSS group (p=0.002). Therefore, it may be concluded that both AE and bovine collagen scaffold were able to stimulate the healing process that will be discussed later for the possible mechanism(s) in the next session.

C. The effect on the re-epithelialization

The process of re-epithelialization in chronic cutaneous ulcer and burn wounds is an important process for complete wound closure (as reviewed previously in Chapter 2 (Figure 2.2)). In normal process of wound healing, epithelialization represents the final state of proliferative phase which occurs during day 3 to day 14. In our study, the process of re-epithelialization was accessed on day 7 and day 14. The re-epithelialization was not significantly different between all groups on day 7 (Table 4.5 and Figure 4.5). This re-epithelialization increased significantly on day 14, in particular, in W+AE0.03, W+Coll and W+Coll+AE0.3, compared to W+NSS group (Table 4.6 and Figure 4.6). This re-epithelialization should be influenced by bovine collagen scaffold. In fact, collagen is accounted as an important factor of extracellular matrix formation that extracellular matrix is an ultrastructure that supplies a substratum for cell adhesion. It also helps critically to regulate the growth, movement, and differentiation of cells through its networking. The present result provides an evidence to indicate the roles of bovine collagen scaffold in cell migration. The results on day 14 indicated that W+AE0.03, W+Coll-group, and W+AE0.3 with bovine collagen scaffold effectively enhanced re-epithelialization up to 100%. A mechanism underling these observations may be associated with their effects on angiogenesis which is the other important process required for wound heeling and it exists in the phase of proliferation similar to re-epithelialization. For further detail it will be discussed later in the following section.

D. The effect on the angiogenesis

Our obtained results of capillary vascularity (%CV) in Table 4.7 and Figure 4.7 showed that %CV of W+AE0.03 and W+Coll+AE0.3 were significantly increased as compared to W+NSS group as early as day 7. However, the %CV of W+Coll+AE0.3 group significantly increased more than W+NSS group on day 14 (Table 4.8 and Figure 4.8). Therefore, it may be noted that both AE and bovine collagen scaffold could stimulate wound neovascularization.

By using biodegradable scaffolds implant in dorsal skinfold chambers of mice, Rucker et al. (2006) showed that bovine collagen scaffold could support angiogenesis or new blood vessel growth. The collagen scaffold is a porous biocompatible material that can behave as an ultra-network for fibroblasts and vascular endothelial cells from the surrounding tissue to migrate into and then replace it with a matrix that resembles normal dermis. At present, there are several trials that aim to make more efficacies for collagen scaffold by adding exogenous growth factors with collagen scaffold to accelerate wound healing; in particular growth factors liked VEGF and bFGF.

There are several reports indicated that one bioactive compound of AE, known as β–sitosterol, could act as an effective angiogenic factor (Eun-Joung Moon.,et al, 1999 and Seongwon Choi., et al, 2002). Since β–sitosterol can be dissolved by ethanol, therefore, our AE ethanol extract should definitely contain some of β–sitosterol, estimated equal to 0.024%.

Therefore, the possible explanation for the most effective result of 14-day Coll+AE0.3 on enhancing wound-angiogenic process may be contributed by both roles of bovine collagen and AE's active ingredient as described above.

E. The effect of the tissue VEGF level

Vascular endothelial growth factor (VEGF) is a growth factor to induce angiogenesis. Normally, VEGF expresses at low level in the skin, however, in wound the production of VEGF was be stimulated by many factors (Szpaderska et al, 2005). As shown in Table 4.8 and Figure 4.8, W+AE was effectively increased capillary vascularity.

We further investigated whether this effect would be attributed through increasing VEGF or not.

The present results showed no significant difference between all groups on day 14 (Tables 4.9-4.10 and Figures 4.9-4.10). However, on day 7, tissue VEGF level significantly increased in W+Coll+AE0.3 (63.81±3.25 pg/ml), compared to W+NSS (48.33±6.11 pg/ml). β-sitosterol is contained in AE ethanol extract as mention above. Accordingly to Seongwon Choi., et al, β-sitosterol in AE extract could enhance angiogenesis by increasing VEGF, and VEGFR (Flk-1) *in vitro.*

F. **The correlation between wound closure and capillary vascularity**

Several studies report that angiogenesis is an essential process for wound healing (Knighton et al. 1990; Brem et al. 1997). In Figure 5.2 and Figure 5.3, the correlations between capillary vascularity (x is %CV) and wound closure (y) were well correlated with best-fitted by a linear line, y= 12.041x-110.6 (r^2=0.969) and y= 6.9557x-58 (r^2=0.98) respectively in W+Coll and W+Coll+AE0.3 groups respectively.
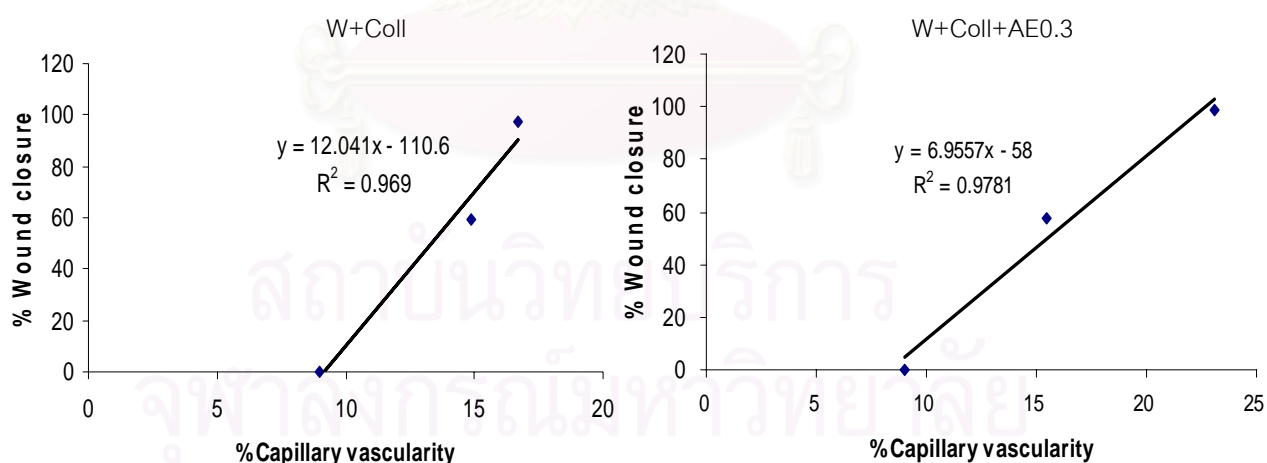

Figure 5.2 In W+Coll group, the correlation between means of capillaryl vascularuty (%CV) and means of wound closure was significant and described by a linear line, y= 12.041x-110.6

Figure 5.3 In W+Coll+AE0.3 group, the correlation between means of capillaryl vascularity (%CV) and means of wound closure were significant and described by a linear line, y= 6.9557x-58 (r^2=0.9781).

In the others groups, the similar correlation testing was conducted and the results were summarized and shown in Table 5.1. It could be noted that the good fitted linear correlation was obtained only in groups of W+Coll and W+Coll+AE0.3. The reason for explaining this finding might be attributed by common properties of bovine collagen scaffold on supporting cell proliferation and by bioactive compound, β-sitosterol in AE extract. Furthermore, this kind of correlation also demonstrated us as a good evidence that the W+AE (0.3 g/kg.bw) - extract enhanced angiogenesis helps bovine collagen scaffold to promote rapid wound healing.

Table 5.1 Correlation factors (R^2) and linear regression lines (Y) between means of percent capillary vascularity (%CV) and means of wound closure were summarized for every groups.

Group	Y	R^2
W+NSS	0.0993x+44.964	5.00E-06
W+AE0.03	2.8185x+12.0508	0.0822
W+AE0.3	9.5296x - 82.303	0.5289
W+Coll	12.041x - 110.6	0.969
W+Coll+AE0.03	12.396x - 101.73	0.5626
W+Coll+AE0.3	6.9557x - 58	0.9781

G. Possible mechanisms for enhancement of the wound healing processes.

In the full-thickness wound, wound homeostasis stimulates the inflammatory response, leading to the proliferative phase. In this phase, angiogenesis occurs by VEGF stimulation, leading to increase in blood nutrition, oxygen supply to the wound tissue, associated with cell migration of keratinocytes at the wound edge and high levels of VEGF expression. These suggest that during wound healing, angiogenesis may be stimulated by keratinocyte-derived VEGF (Brown et al. 1992; Ballaun et al. 1995; Detmar et al. 1995; Frank et al. 1995).

Angiogenesis is an essential process for wound healing. Newly formed vessels provide blood supply promptly to wound area during the healing (Knighton et al. 1990). Since angiogenesis is conducted in association with the proliferative phase, it may enhance and initiate cells growth after wound generation (Tonnesen et al. 2000). The angiogenesis process may be stimulated by both homeostasis and inflammation which produces cytokine, chemokine and many growth factors to enhance new blood vessels. In addition, wound hypoxia occurred at the early phase of inflammation, inducing hypoxia inducible factor (HIF)1-α. The HIF1-α could further induce angiogenic growth factor as VEGF.

In general, full-thickness wound presents a number of clinical problems such as infection, loss of fluid and the scar formation. Bacteria are a microorganism to stimulate the infection, inflammation, leading to the delay of wound healing. Neutrophils are initial leukocytes that come into the wound tissue to clear the pathogenic, bacteria or virus. The number of neutrophil can indicate the level of some pathogen into the wound. The lack of nutrition and oxygen is a problem of full-thickness wound in that the vessels are destroyed, leading to the wound-healing delay.

This research was also designed to resolve the problem of the wound healing delay by using bovine collagen scaffold with AE extract. We discussed the effectiveness of the bovine collagen scaffold and this AE in the above section. Let us discuss about the wound healing delay. Bovine collagen scaffold may be behaved as extracellular matrix for cell migration, inducing the angiogenesis for better healing of the wound tissue. In the present experiment, in W+Coll group and W+Coll+AE groups, the wound

closure significantly increased more than the control (W+NSS) group. In addition, the W+Coll+AE0.3 showed that capillary vascularity was more than that in W+NSS group. This indicates that the supply of blood and nutrition to the wound is increased, leading to good conditions for wound healing. The VEGF level measured in tissue demonstrated higher level of capillary vascularity in W+Coll+AE0.3. Moreover, re-epithelialization increased in W+Coll+AE0.3 group, indicating that the cell migration into the wound increases angiogenesis, leading to stimulation of wound healing.

In W+Coll+AE0.3, neutrophil infiltration decreased on day 3, suggesting that the inflammation process was reduced. This confirms the effect of W+AE, 0.3 g/kg bw, combined with bovine collagen scaffold can lead to the improvement of wound-healing.

It is concluded that the daily topical-treatment of W+AE (0.3 g/kg bw) could enhance the beneficial effects of bovine collagen-scaffold implanted in full-thickness wound. Possible mechanisms underlying the wound healing might be attributed by the combined effects of bovine collagen and AE, including decease in the wound-inflammation, increase in VEGF expression, stimulation in the angiogenesis, and re-epitheliaization.

Suggestion

Although, the result of this study indicated that W+Coll+AE0.3 could enhance rapid wound healing in associated with the increased VEGF expression and consequently increased capillary vascularity. However, further research on the possible mechanism of AE is needed. β-sitosterol in AE extract might not be only one kind of active ingredients in AE that was responsible for stimulating VEGF expression during proliferative phase in wound-heeling process.
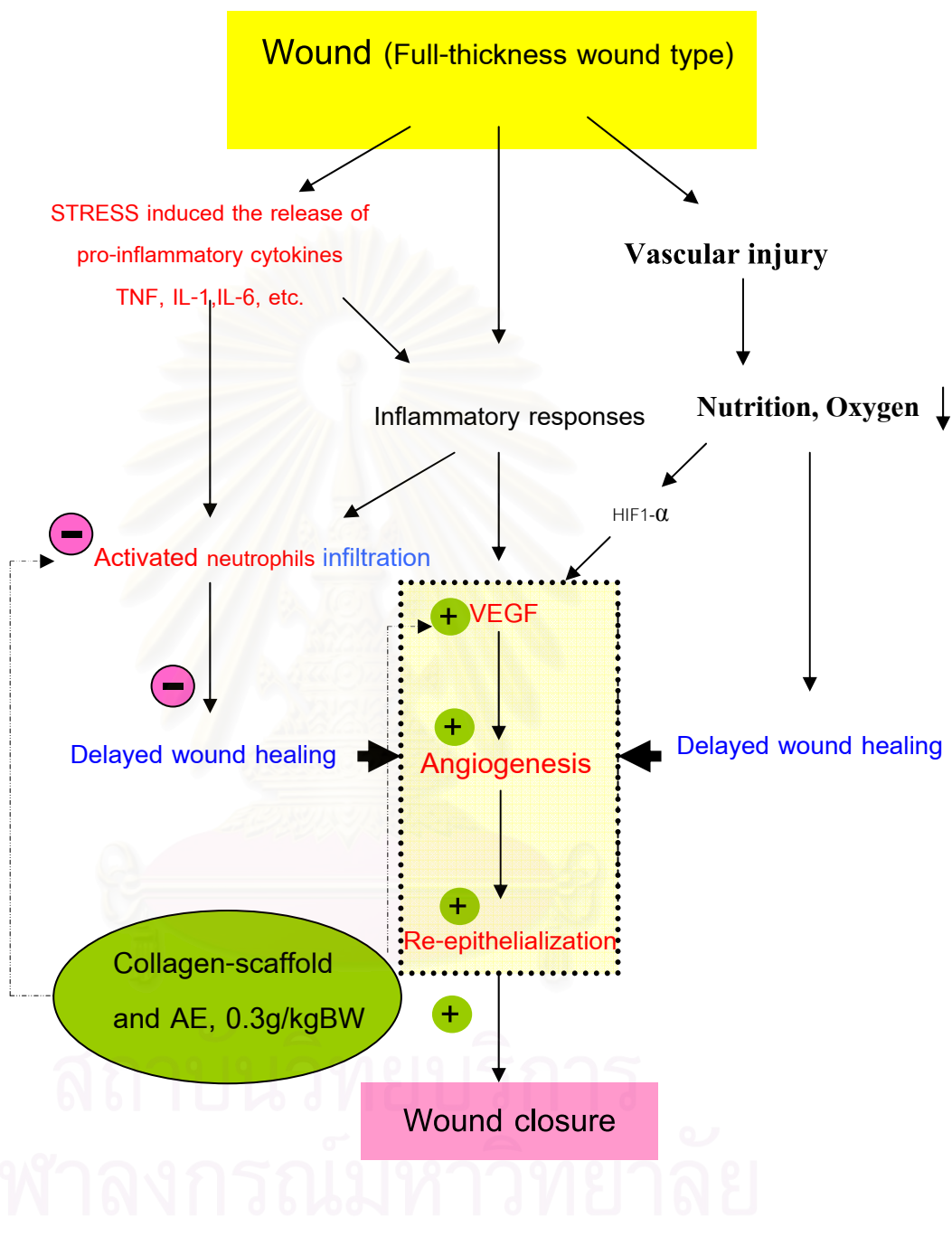
Figure 5.4 The proposed mechanism of bovine collagen scaffold combined with *Acanthus Ebracteatus* Vahl. Extract (dose = 0.3 g/kg bw) on enhancing angiogenesis and wound closure in full-thickness wound-mice model.

CHAPTER VI

CONCLUSION

This investigation of the overall results showed the combination of bovine collagen scaffold and AE0.3 for treatment of full-thickness wound as follows:

1) W+Coll+AE0.3 could improve wound healing by anti-inflammation via reduction of neutrophil infiltration. In addition, the effects were due to induce in re-epithelialization and increase in new capillaries. These were two factors of wound closure or recovery.

2) The effectiveness of bovine collagen scaffold combinaed with *Acanthus ebracteatus* Vahl. 0.3 g/kg.bw. on full-thickness wound in mice, were demonstrated. To show benefit further investigation for its mechanism will be need.

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