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EVALUATION OF WOUND HEALING ACTIVITY OF THUNBERGIA LAURIFOLIA EXTRACT IN THE SECOND DEGREE BURN WOUND RATS

Mrs. Juthaporn Kwansang

A Dissertation Submitted in Partial Fulfillment of the Requirements
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Thesis Title	EVALUATION OF WOUND HEALING ACTIVITY OF THUNBERGIA LAURIFOLIA EXTRACT IN THE SECOND DEGREE BURN WOUND RATS
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การศึกษานี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ในการรักษาแผลไหม้ของสารสกัดจากใบรางจืด โดยใช้ วิธี supercritical CO_2 ทำการศึกษาในหนูแรทพันธุ์วิสตาร์น้ำหนัก 200-250 กรัมที่ถูกเหนี่ยวนำให้เกิดแผลไหม้ระดับสอง แบ่งหนูแผลไหม้โดยวิธีสุ่มเป็น 6 กลุ่ม กลุ่มละ 6 ตัวแต่ละตัวได้รับการทายาเฉพาะที่ 200 มิลลิกรัมวันละครั้งบริเวณแผลไหม้เป็นเวลา 14 วัน ประกอบด้วยเจลพื้นฐาน, 1% ซิลเวอร์ซัลฟาไดอะซีน, เจลรางจืดความเข้มข้น 2.5, 5, และ 10% และกลุ่มแผลไหม้ที่ไม่ได้รับสารใดๆ ประเมินผลการรักษาแผลไหม้จากเปอร์เซ็นต์การปิดของแผล การเปลี่ยนแปลงทางพยาธิสภาพ การวัดปริมาณคอลลาเจน และ การวัดปริมาณของ TNF-α, VEGF, และ COX-2 ในวันที่ 3 และ 7 หลังเกิดแผลไหม้ โดยวิธีอิมมูโนฮีสโตเคมมีสทรี ผลการทดลองพบว่าค่าร้อยละการปิดของแผลไหม้ในหนูกลุ่มที่ได้รับเจลรางจืดความเข้มข้น 10 % สูงกว่าหนูกลุ่มเจลพื้นฐาน, 1% ซิลเวอร์ซัลฟาไดอะซีน, เจลรางจืดความเข้มข้น 2.5 และ 5% ปริมาณคอลลาเจนซึ่งเป็นตัวบ่งชี้การสร้างเนื้อเยื่อใหม่ของแผลใน proliferative phase ของหนูกลุ่มได้รับเจลรางจืด 10% เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติในวันที่ 7 และ 14 ภายหลังได้รับยาเมื่อเทียบกับกลุ่มแผลไหม้ ซึ่งสอดคล้องกับปริมาณเส้นใยคอลลาเจนสีฟ้าที่พบจากการย้อมสี Masson's trichrome และกลุ่มที่ได้รับเจลรางจืด 10% มีอาการบวม และเซลล์ที่เกี่ยวข้องกับการอักเสบลดลงอีกทั้งยังพบว่ามีการเพิ่มจำนวน ของ hair follicle และการสร้างเซลล์ epithelium เพิ่มมากขึ้น ยังมีสิ่งสนับสนุนจากการตรวจพบการเพิ่มปริมาณของ angiogenic factor (VEGF) และ การเปลี่ยนแปลง proangiogenic factors (TNF-α) ร่วมกับการลดลงของ COX-2 enzyme ผลการทดลองข้างต้นแสดงถึงศักยภาพในการรักษาแผลไหม้ของสารสกัดรางจืด

สาขาวิชา เภสัชวิทยา ลายมือชื่อนิสิต .....

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JUTHAPORN KWANSANG: EVALUATION OF WOUND HEALING ACTIVITY OF THUNBERGIA LAURIFOLIA EXTRACT IN THE SECOND DEGREE BURN WOUND RATS. ADVISOR: ASSOC. PROF. CHANDHANEE ITTHIPANICHPONG, CO-ADVISOR: ASST. PROF. WACHAREE LIMPANASITHIKUL, Ph.D., 102 pp.

The aim of this study was to investigate the burn wound healing activity of supercritical CO_2 *Thunbergia laurifolia* (TLL) leaf extract in second degree burn wound rats. Male Wistar rats weighing 200-250 g were used and divided into six groups. There were six animals in each group. They were treated topically with 200 mg gel base, 1% silver sulfadiazine (SSD) gel, 2.5%, 5%, and 10% TLL gel at wound area for 14 days. Evaluation of wound healing activity included an estimation time of wound closure, collagen content, histopathology, and immunohistochemistry analysis of the wound tissue. The results revealed that the percentage of wound closure in 10% TLL gel treated group was higher than those observed in gel base, 1% SSD, 2.5, and 5% TLL gel treated groups. The collagen content which is a marker of tissue regeneration in proliferative phase was also significantly increased in 10% TLL treated group at day 7 and 14 post burning compared to untreated burn group. This result was also confirmed by Masson's trichrome tissue analysis which increased collagen content in blue color. Reduction in edema, congestion, and inflammatory cells along with increased number of hair follicles, and epithelialization were clearly observed in 10% TLL treated groups. Accelerating of wound healing effect of 10% TLL group was further supported by increasing of angiogenic factor (VEGF) and altering of proangiogenic factor (TNF-α) in accordance with a reduction of COX-2 enzyme. These data clearly illustrated the therapeutic potential of *T. laurifolia* in treating burn wound.

Field of Study: Pharmacology
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Student's Signature .....
Advisor's Signature .....
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CONTENTS

Page

Page

Page

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BW	Body weight
cm	Centimeter
SC-CO_2	Supercritical carbon dioxide
COX-1	Cyclooxygenase-1
COX-2	Cyclooxygenase-2
°C	Degree Celsius
DAB	Diaminobenzidine
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
IL-1	Interleukin-1
IL-6	Interleukin-6
IP	Intraperitoneal
G	Gram
HPTLC	High performance thin layer chromatography
HRP	Horseradish peroxidase
Kg	Kilogram
MIC	Minimum inhibitory concentration

min	Minute
mg	Milligram
ml	Milliliter
nm	Nanometer
NO	Nitric oxide
PDGF	Platelet derived growth factor
PG	Prostaglandin
PO	Per oral
SC	Subcutaneous
SD	Standard deviation
SFE	Supercritical fluid extraction
SPSS	Statistical package for the social sciences
TBS	Tris-buffered saline
TNF-α	Tumor necrosis factor-alpha
TGF-β	Transforming growth factor-β
TLL	*Thunbergia laurifolia* Lindl.
µg	Microgram
µl	Microliter
µm	Micrometer
UV	Ultraviolet

VEGF	Vascular endothelial growth factor

CHAPTER I

INTRODUCTION

Background and Rationale

Thermal burn is commonly induced in tissues by an accidental exposure of excessive thermal energy including fire, scald, chemicals, electrical current and radiation (1). Thermal burn induces cellular damage primarily by transfer of energy that induces coagulation necrosis, while direct injury to cellular membranes is the cause of injury. The body responds to burn by releasing of inflammatory mediators such as prostaglandins, nitric oxide, cytokines, enzymes in the wound and other tissues (2). These mediators increase capillary permeability and edema locally. They start the wound healing process as well.

Wound healing is a complex process involving inflammatory, proliferative, and remodeling phases. Neutrophils and macrophages are important cells in the inflammatory phase. The activated macrophages release inflammatory cytokines and enzymes responsible for inflammation such as TNF-α, IL-1, IL-6, and COX-2. They also promote tissue repair by increasing growth factors release including PDGF, VEGF etc.
In the proliferative phase macrophages and fibroblasts predominantly produce growth factors that play important roles in the wound healing process. Fibroblast is activated by cytokines like transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), and vascular endothelial growth factor (VEGF) to produce collagen, increase granulation tissue, induce angiogenesis and epithelialization. Angiogenesis is a process of new blood vessel formation increasing proliferation and migration of endothelial cell. VEGF increases proliferation and migration of keratinocytes in wound edge supporting wound closure.

In remodeling phase, there is a balance between collagen synthesis and collagen degradation. The maximum collagen synthesis is found in 3 weeks after wound injury (3-5).

Management of burn wound depends on the depth and area of burn which are classified into first, second, third and fourth degree (2). The first and second degree burn can reverse to normal skin by itself within 1-2 weeks after burn. However the burn wound healing can be accelerated through applying topical antimicrobial agents such as silver sulfadiazine (SSD), mafenide acetate (3). The third and fourth degree burn need prolong treatment and require some procedures like excision and grafting to support wound heal (2). SSD is recommended for the treatment of superficial and deep dermal burns (6) but evidences regarding its usefulness in burns is poor. Aziz et al. (2011) found that topical silver may worsen healing time compared to control and showed no evidence of effectiveness in preventing wound infection (7). At present, trend in worldwide for disease treatment has turned to use natural substances (8). Therefore increasing numbers of herbal medicine investigations for the treatment of commonly found diseases are present (9). There are many medicinal plants resembling ideal wound healing agents are recommended by worldwide authorities. These medicinal plants include *Aloe vera*, *Centella asiatica* (10-12). They are used in many developing countries for burn wound treatment because of their effectiveness and low cost.

Thunbergia laurifolia (T. laurifolia), commonly known in Thai as Rang Jued is a herbaceous climber with flowers in purple color. It is generally used as an antidote for people and animals pertaining to toxic plants or chemicals. In Malaysia, the juice from crushed leaves is taken for relieving the symptom of dysmenorrhea, ear deafness and it is also applied as a poultice for cuts and boils (13, 14). The leaves of *T. laurifolia* possessed antioxidant, anti-inflammatory, anti-drug addiction and anti-diabetic (15, 16). However, there has not been found any reports on burn wound healing activity of *T. laurifolia*. Therefore, it is of our interest to elucidate its effects.

Objectives

1. To investigate wound closure effect of supercritical CO_2 *T. laurifolia* leaf extract in rats with second degree burn wound.
2. To investigate the effect of supercritical CO_2 *T. laurifolia* leaf extract on inflammatory mediators, pro-inflammatory cytokines, growth factors and collagen content in rats with second degree burn wound.
3. To investigate the effect of supercritical CO_2 *T. laurifolia* leaf extract on histopathology of the rat tissues with second degree burn wound.

Keywords

Thunbergia laurifolia, supercritical CO_2, wound healing

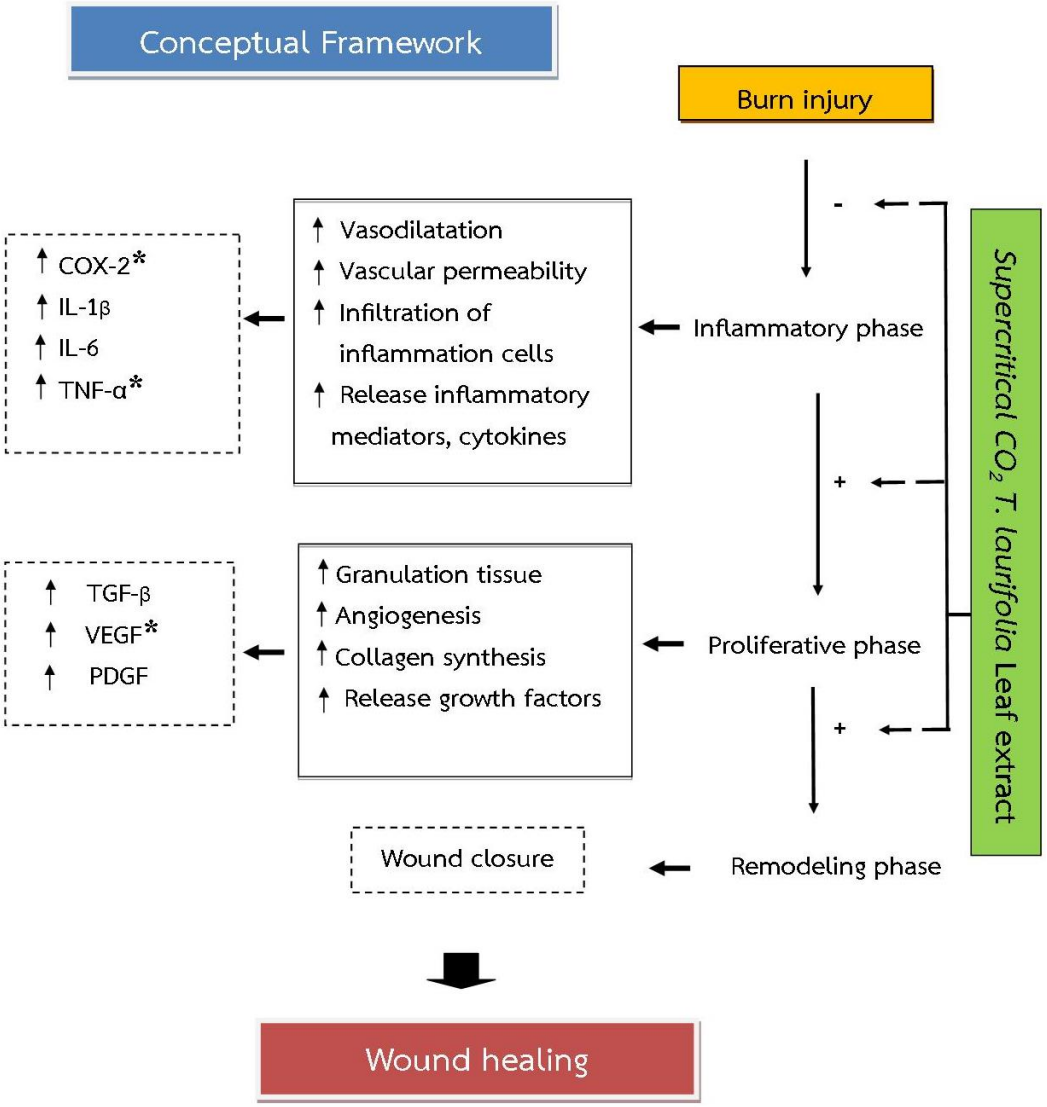


Figure 1. Conceptual framework

* Mediators and enzymes determined in this study

CHAPTER II

LITERATURE REVIEWS

2.1 The skin

The skin is the largest organ in the body, making up 60% of the body's weight and has a surface area of around 1.8 square meters. It has an essential role in homoeostasis and protection from external environment (17).

2.2 Structure of skin

The skin composes of three layers including epidermis, dermis, and subcutaneous layer (18). It is characterized by a tough keratinized surface which protects underlying tissues from the external environments. The thickness of skin varies depending on its location on the body (17). The structure of the skin is presented in figure 2.

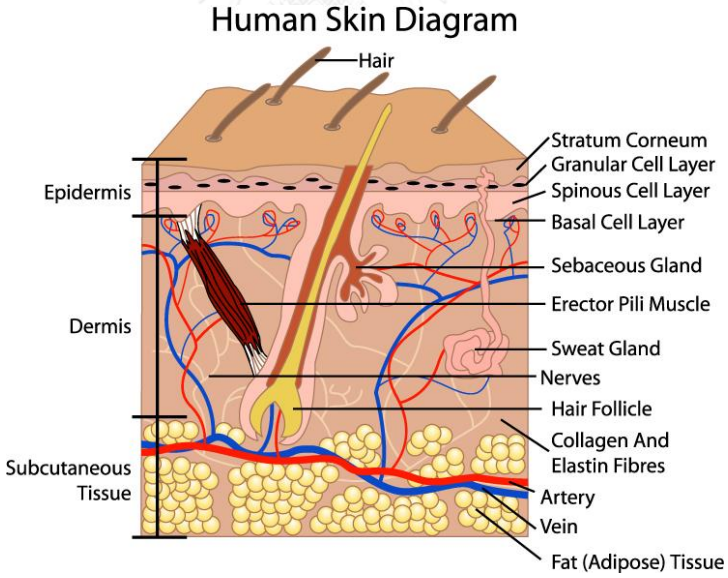


Figure 2. Structure of skin (19).

2.2.1 Epidermis

Epidermis is the outer layer of skin. It consists of the following layers (18):

- Stratum basale
- Stratum spinosum
- Stratum granulosum
- Stratum corneum

The four epidermis layers are formed by keratinocytes that progress through their different stages of maturation to form the protein keratin.

The cells existing in epidermis layers include keratinocytes, melanocytes, Merkel's and Langerhans's cell. Keratinocytes produce keratin which gives the skin its strength and flexibility. Melanocytes produce a dark pigment of skin called melanin. Merkel's cells are involved with touch perception and Langerhans's cells facilitate removal of foreign bodies via immune system.

Stratum basale

This layer is firmly anchored to the underlying basement membrane. It is composed of 90 % keratinocytes, 5-10% melanocytes and infrequent Merkel's cells. Basal cells are the skin stem cells which are differiated to replace superficial keratinocytes shed at epithelial surface. They can be visualized on electron microscopy. The stratum basale forms epidermal ridges that extend into the dermis, increasing contract between the two layers. It is highly developed in the areas exposed to external environment such as the hand and feet.

Keratinocyte function

The main functions of keratinocytes are producing the protein called keratin. The stages of keratinocyte maturation have been described. Each different stages of maturation produce different molecular weight keratins. Hence, different keratinocytes are found in each separate layer of the epidermis. As well as producing keratin, keratinocytes also synthesize pro-inflammatory cytokines, such as interleukin-1 (IL-1), some growth factors (e.g. VEGF) that are important messengers in the inflammatory and proliferative phase (17).

Stratum spinosum

This layer, keratinocytes are divided by mitosis from stratum basal and they migrate upwords forming the stratum spinosum. This layer also contains Langerhans's cells which migrate and bind penetrating environmental antigen, that presenting them to lymphocytes in a mechanism participating in the systemic immune response.

Stratum granulosum

In this layer, keratinocytes produce keratohyalin and keratin granules. As keratinocytes begin to mature, the cells flatten, lose their intracellular organelles nuclei and die. Disintegration of their intracellular content causes them to dehydrate resulting in a tough, impermeable layer of keratin formation.

Stratum corneum

This layer contains 15-30 layers of keratinized, overlapping cells with lacking of nuclei, glued together tightly by desmosomes. Keratin provides flexibility and strength of skin. The stratum corneum is water resistant. Interstitial fluid is lost through the stratum corneum in the process of insensible perspiration.

2.2.2 Dermis

Dermis is a layer of skin between the epidermis and subcutaneous tissue. It consists of two layers: the superficial papillary layer and the underlying reticular layer. The papillary layer contains capillaries and sensory nerves supplying the skin surface which embedded in loose connective tissue. The reticular layer contains bundles of collagen fibers that anchor the papillary layer to the underlying subcutaneous layer. In addition, the dermis contains mast cell and specialized nerve ending as well as vascular plexuses, hair follicles and sweat glands (20).

Hair follicle

A hair follicle is a mammalian skin organ. It represents complex and dynamic three dimension structures as shown in Figure 3. There are approximately 5 million hairs on the body, 98% of which are located on the skin surface. Hairs can find in a varying density over the entire surface except on the palms and soles. Hairs emerge from follicles which most densely distributed on the scalp and face. Scalp hair provides

some protection against the harmful effects of ultraviolet radiation and minor injuries (17).

Structure of hair follicle

A hair follicle consists of hair papilla, hair matrix, hair bulb, hair root, and hair shaft. The base of the hair follicle is the hair papilla which is a structure formed from the connective tissues that hold capillaries and nerves supply. Around the papilla is the hair matrix which function is producing the cells for the major structures of the hair fiber and inner root sheeth. Bulb is a part of hair follicle located in the outer root sheath. It contains several types of stem cells that supply new cells for the entire hair follicles and plays role in healing the epidermis after wounding.

The hair shaft is formed of three layers:

1. The inner medullar or core contains flexible keratin.
2. The outer cortex contains hard keratin and gives a hair its stiffness.
3. The cuticle layer is a protective outer most layer. It protects the safer inner structure including the medulla and cortex. It also makes a hair shiny (17, 18, 21-23).

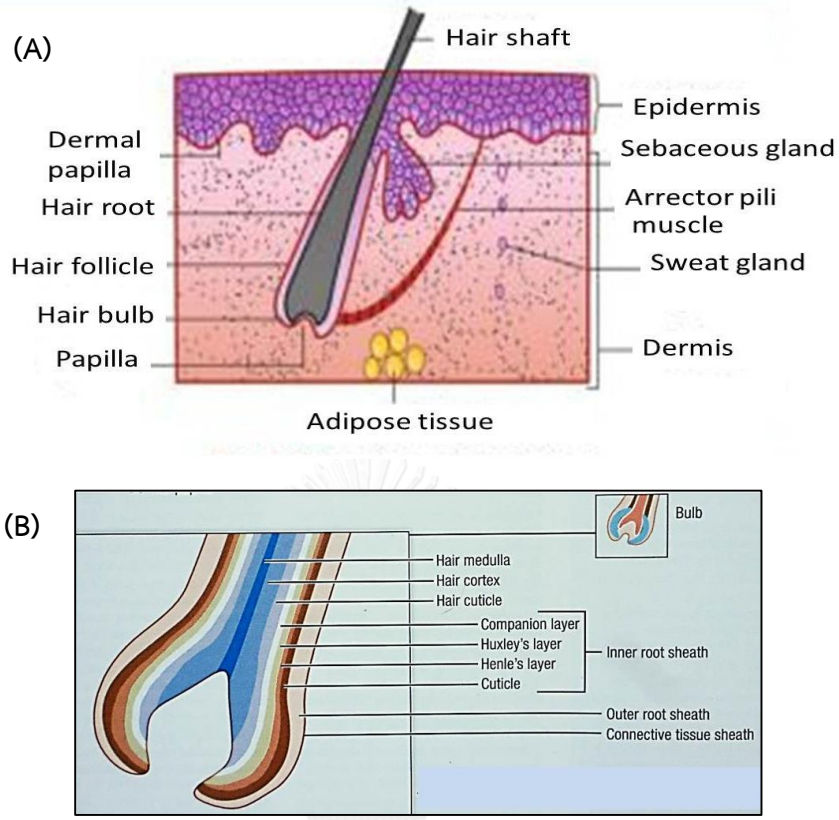


Figure 3. A and B are structure of hair follicle (18, 24)

Hair follicle and wound healing

Hair follicles play part in healing of injured skin. Wound removes the epidermis surface and hair follicles. The deep wounds destroy the hair follicles and delay to heal (25). A major component of the wound healing process is the replacement of an epidermal barrier by growth and migration of continuous epithelial cells to the wound and by migration of hair follicle cells to skin surface (26). Hair follicles are important to wound healing because they contribute epidermal cells for resurfacing partial-thickness wound such as the second-degree burn wound which absences of hair follicle. It is a factor contributing in the prognosis of wound healing (20).

2.2.3 Subcutaneous layer

The subcutaneous layer mainly consists of fat. It is collectively referred to the adipose tissue. It is important in maintaining the stability of the skin in relation to its underlying tissues and structures (17).

2.3 Function of the skin

Skin performs several functions, as the following:

1. Mechanical barrier to infection, toxins, antigens and external insults
2. Contributing to thermoregulation
3. Maintaining cutaneous immunity and coordinating wound healing responses
4. Synthesizing vitamin D within the epidermis on exposure to sunlight
5. Protecting against excessive water absorption or loss
6. Protecting, via skin pigmentation, against ultraviolet (UV) light
7. Providing and mediating sensation, distinguishing between pain, touch, pressure and temperature modalities

2.4 Wound

Wound defines as the loss of continuity of epithelium with or without the loss of connective tissue, nerves, muscle, and bone. Wounds are classified into several types including abrasion wound, laceration wound, incision wound and burn wound (27).

2.4.1 Abrasion wound

Abrasion wound is a wound caused by damage of the skin, no deeper than the epidermis. It also knows as grazes or scrapes which do not scar or bleed. It is commonly caused by a glancing impact across the surface of the skin.

2.4.2 Laceration wound

Laceration wound is known as tears or cuts of the skin. The skin surface is split or torn following blunt trauma and the force causes the full thickness of the skin to damaged. Laceration wound has ragged wound edges, torn apart, and not neatly incised as in a surgical wound.

2.4.3 Incision wound

Incision wound is sharp cut-like injuries. The edges of the wound will vary according to the nature of the cutting edge of the objects. Incision wound is divided into two types, surgical and non-surgical incision wound. Surgical incision wounds are clean whereas non-surgical wound caused by injuries inflicted by a knife or other sharp instrument.

2.4.4 Burn wound

Burn wound is an injury caused by energy within a tissue resulting in a disruption of functional integrity. The source of the energy are thermal, chemical, electrical and radiation (28).

2.5 Pathophysiology of burn

The skin generally provides a barrier to limit transfer of energy to deeper tissues. After the source of burn is removed, the response of local tissues can lead to further injury. Burn injury causes protein denaturation, damage capillary endothelial cells. Burn wound also attributes to the secondary consequences of burn injury such as edema, infection, and altered perfusion which promotes progression of injury (29, 30). Burn induces disruption of collagen cross-linking and abolishes the integrity of osmotic and hydrostatic pressure gradients, resulting in local edema and larger scale fluid shifts. At

the molecular level, both complement activation and intravascular stimulation of neutrophils result in the production of cytotoxic oxygen free radicals, increased histamine activity, and activated secondary cytokines (1, 31).

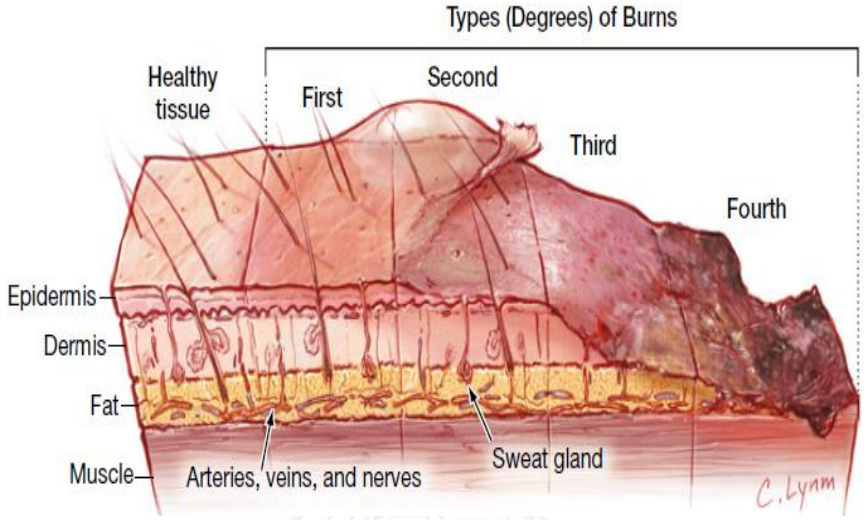


Figure 4. Burn wound classification by burn depth (33)

2.6 Classification of burn depth

Burn injuries cause cell death from traumatic and ischemic necrosis. The depth and severity of burn are depended on temperature, concentration and duration of contact with the causing agents (32). Burn wounds have been described as first, second, third and fourth degree (1, 3, 33).

Table 1. Burn wound classification by depth (3)

Degree of burn	Depth	Wound appearance	Treatment	Healing
First-degree	-Epidermis	-Erythematous Slight red	-Topical agents	3-7 days
Second-degree	-Superficial partial-thickness; Epidermis and dermis (papillary dermis)	-Erythematous (red to pink) - Blister formation	-Topical agents (with bacteriocidal)	7-14 days for superficial
	-Deep partial-thickness; Epidermis and dermis (reticular dermis)	-Pale (red or white) -Blanching absent or prolonged -Edematous -Decreased moistness	-Topical agents (with bacteriocidal) -May require skin grafting	21-28 days or longer
Third-degree	-Epidermis, dermis, and epidermal appendages	-Dry surface -Pale and mottled (brown, white or red) -Visible thrombosed vessels	-Early excision and grafting	Require excision and grafting to heal
Fourth-degree	-Epidermis, dermis, connective tissue, muscle and bone	-Charred and mottled (brown, white or red) -Dry surface	-Early excision and grafting -Likely amputation of involved extremities	Require excision and grafting to heal

2.7 Burn wound treatment

The goals of burn wound care are prevention of infection and wound healing promotion. It is depended on the healing status of wound such as location, size, and depth of the burn. In minor burn, the patient should be removed the dead skin, open blister, and apply topical antimicrobial agent. For major burn, the patient needs transferring to the specialist for skin grafting and taking care in an intensive unit. Usually topical antimicrobial agents are used for prevention of infection. The topical antimicrobial agents for burn wounds are summarized in table 2.

Table 2. Topical antimicrobial agents for burn wounds (3)

Agents	Mechanism of action	Advantages	Disadvantages
Mafenide acetate 11.1% water-solution cream	Bacteriostatic for gram-positive bacteria and gram-negative bacteria	-Broad-spectrum topical antibiotic and penetration into burn eschar	-Painful application - No fungal coverage
Silver nitrate 0.5% solution	Broad spectrum anti-microbial activity	-Painless application -Broad-spectrum of activity including fungi	-Difficult to apply -Poor eschar penetration
Silver sulfadiazine (SSD), 1% water-soluble cream	Silver affects to multiple sites within bacteria cells that causing disruption of the bacterial cell. Sulfadiazine works by inhibiting the growth and replication of bacteria.	-Well tolerated -Painless application	-Contra-indicated in patients with sulfa allergy -Leukopenia -Skin discoloration -Rarely, erythema multiforme and interstitial nephritis

2.8 Wound healing process

There are 3 phases in wound healing processes comprising of

1. Inflammatory phase
2. Proliferative phase
3. Remodeling phase as shown in Figure 5.

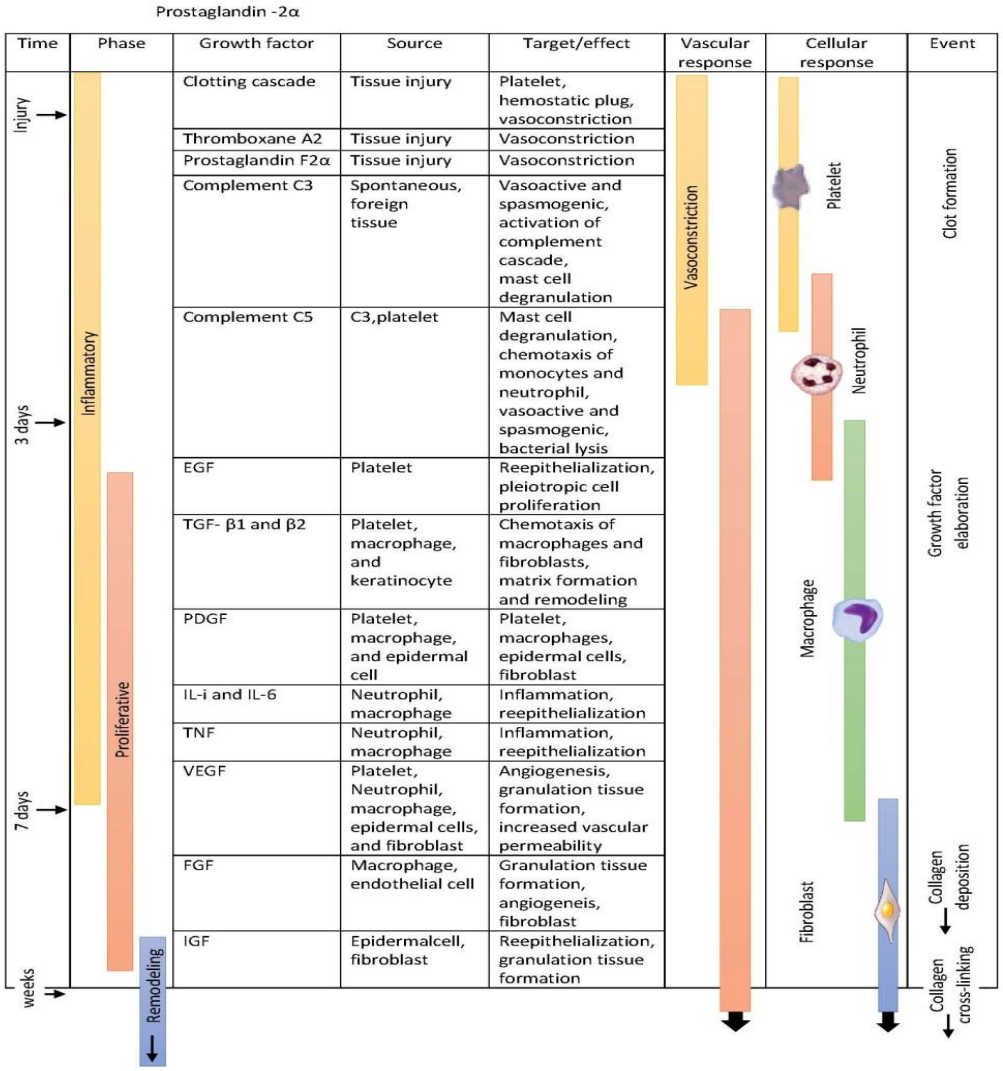


Figure 5. Cytokines and complements involved in wound healing. The three phases of wound healing are associated with different growth factors and subsequent cellular infiltration (34).

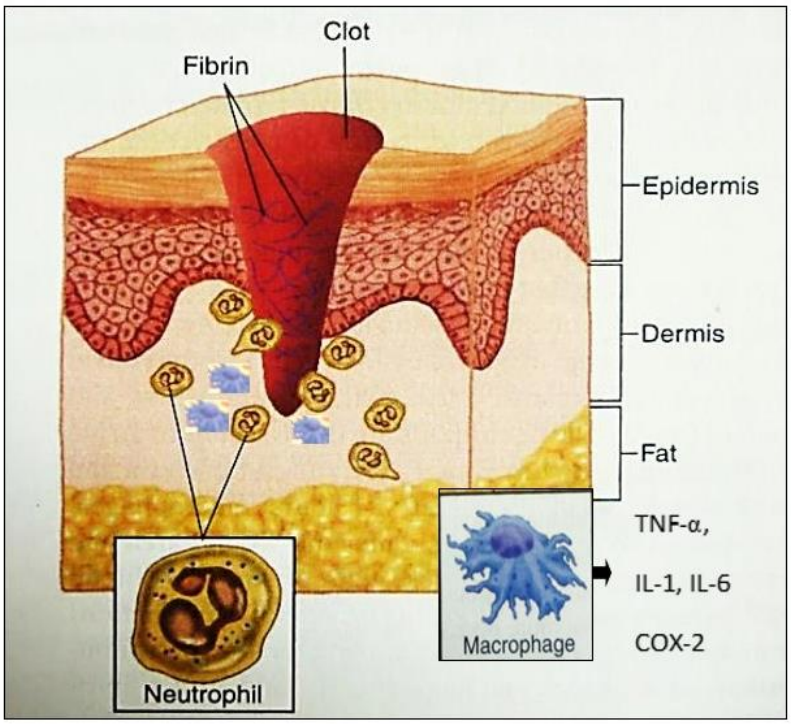


Figure 6. Neutrophils and macrophages are important cells in the inflammatory phase. Activated macrophages produce inflammatory cytokines into the wound tissue (5).

2.8.1 Inflammatory phase

After tissue injury, the body starts up to stop bleeding and progresses in inflammatory phase. Neutrophils and macrophages move into injured tissue. Neutrophils are important cells in the first process of tissue injury and replaced by macrophages later. Activated macrophages produce NO, TNF-α, IL-1, IL-6, and COX-2 as pro-inflammatory cytokines and enzymes contribution to inflammation (35). COX-2 generates a high level of prostaglandins that mediate the inflammatory process, pain and fever. Then macrophages start wound healing process by releasing growth factors, activation of fibroblasts and infiltrating of granulation tissues (36) in Figure 6.

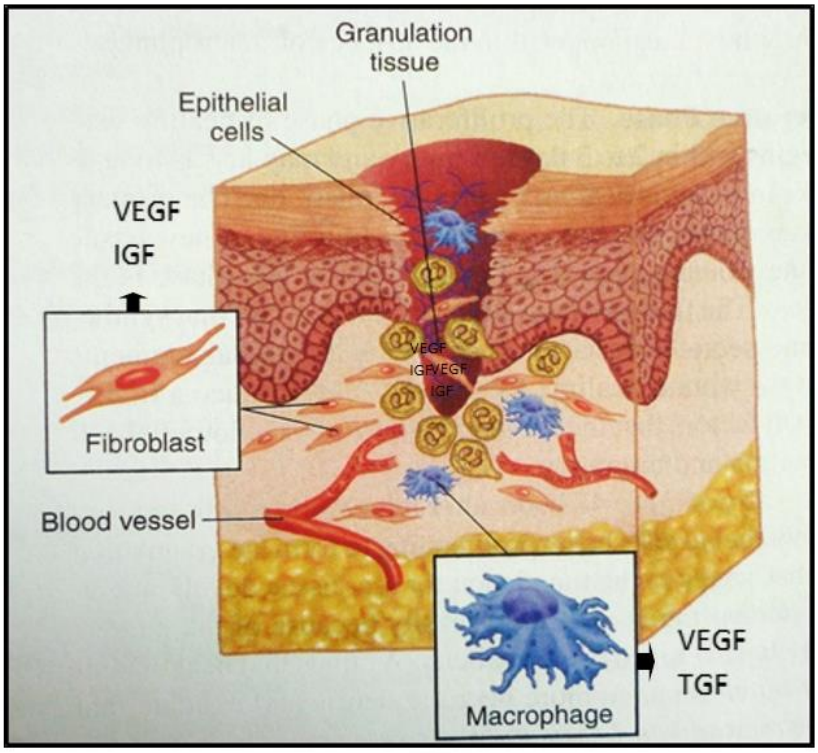


Figure 7. Macrophages and fibroblasts produce angiogenic cytokines, growth factors to promote wound healing in proliferative phase (5).

2.8.2 Proliferative phase

The proliferative phase begins within 2 to 3 days of injury and may last long for 3 weeks in wound healing. Macrophages and fibroblasts are important cells during this phase. Macrophages exert multifunction in wound healing process including chemotaxis, inflammatory cells stimulation, fibroblast activation, promoting angiogenesis, and epithelialization as shown in Figure 7 (4).

Fibroblasts

Fibroblast is a key cell responsible for collagen synthesis and other intercellular elements that needed for wound healing. The main components of the extracellular matrix are collagen, elastin and proteoglycans produced by fibroblasts (17, 37).

Collagen synthesis

Collagen is synthesized by fibroblasts, smooth muscle cells and endothelial cells. It is composed of three polypeptide chains braided into triple helix. Two types of collagen are important in wound healing. Type I collagen predominates the extracellular matrix of normal skin, type III collagen presents in tissue healing. Normal skin contains type I and type III in a 4:1 ratio. Increased amount of type III collagen is found in the early phase of wound healing. Collagen synthesis begins after wound healing by activation of fibroblasts by growth factors and the environment in the wound which begins at day 3 to day 5 after wound burn and continues for several weeks. Thereafter, the amount of collagen type III decreases in relation to type I. Collagen homeostasis is completed when the collagen synthesis and degradation are equivalent, which occurring in three weeks post burn (3, 4, 38).

Angiogenesis

Angiogenesis is a process of new blood vessel formation. It consists of proliferation and migration of endothelial cells toward the area of tissue injury, remodeling into capillary tubes, and recruitment of periendothelial cells to a mature vessel. The process of angiogenesis involves a variety of cytokines and growth factors. Major angiogenic cytokines such as VEGF, TGF-β exert their influence by promoting migration of endothelial cells to the injured site. Nogami et al. (2007) reported that VEGF, fibroblast-like cells, and endothelial cells were found in rat skin incision wound

on day 3 and day 7 after injury in higher amount than in control group (39-41). There has been reported that TNF-α played its role as an angiogenic factors by activating endothelial cell for angiogenesis and TNF-α expression was associated with VEGF expression in early wound healing (day1-4) (4, 42-44).

Granulation tissue

Granulation tissue is a non-permanent tissue that appears only during the injury repair process. It is composed of new blood vessels, fibroblasts and inflammatory cells. The granulation tissue is a symbol of tissue repair. It will be replaced by myofibroblasts in consequence of the cell fibroblast alteration and the aggregation of actin from smooth muscle. This leads to formation of thickness and increasing numbers of fibers in this area, ultimately with a fibrotic scar (45).

Epithelialization

Epithelialization is a process that the epithelial cells from wound edge migrate into the wound bed. Epidermis consists of multiple layers of epithelial cells which are formed by keratinocytes. In wound healing, keratinocytes proliferate and migrate associated with cytokines and growth factors production including IL-1, IL-8, TNF-α, PDGF, VEGF and TGF (46-48). The basal epithelial cells involved in the closure of wounds are derived from both the wound edges and skin appendages including hair follicles, sweat glands and sebaceous glands (49).

2.8.3 Remodeling phase

This phase begins until 3 weeks after injury with the development of scars. The repair process is a balance between synthesis and degradation of extracellular matrix (ECM). ECM is a complex of several proteins such as collagen, elastin, proteoglycan,

etc. that assembles into a network (39). This phase lasts for 6 months or longer, depending on the type, depth, and area of wound. The granulation tissues transfer to scar formation and no new blood vessels formation. The human wound is estimated to reach its maximal strength at one year with maximal tensile strength. The remodeling are still synthesis, and remodeling of the extracellular matrix providing strength to normal skin (3, 34).

2.9 Inflammatory mediators and enzyme

Since, there are many inflammatory mediators, cytokines, growth factors, and enzymes involving in the wound and wound healing process, thus, they are briefly summarized as follows:

2.9.1 Cyclooxygenase-2

Prostaglandins are produced from arachidonic acid by the action of enzyme cyclooxygenase 1 and 2 (COX-1 and COX-2). Both COX isoforms produce prostaglandin H (PGH_2) that is the originated source of other prostaglandins (PGI_2, PGD_2, PGE_2 and TXA_2). It is known that COX-2 expression is not found in normal tissue. However, it can express later by inflammatory and painful activation. Prostanoids are inflammatory mediators and they are secreted in the principal period of inflammation. Their production and functions are depending on each cell types. For example, mast cells possess drastic secretion of PGD_2, macrophages secrete PGE_2 and TXA_2 while endothelial cells release PGI_2 respectively (45). PGE_2 appears to be one of the potent inflammatory prostaglandins causing post burn vasodilation. When coupled with the increased microvascular permeability, it amplifies edema formation (50). In conclusion, all these activities are associated with vasodilation and causing pain. Apart from being an inducible enzyme for generation of inflammatory prostaglandins, COX-2 also

promotes angiogenesis in wound healing by acting as pro-angiogenic factor for VEGF up regulation (51-53).

2.9.2 Tumor necrosis factor-α (TNF-α)

TNF-α is a cytokine elevated broadly from monocytes, macrophages and other cell sources including lymphocytes, mast cells, neutrophils, keratinocytes and smooth muscle cells (4). It is an important mediator in the pathogenesis of trauma, sepsis, and inflammation (54). In wound healing process, TNF-α acts as an inflammatory mediator in the inflammatory phase and stimulates fibroblasts, accelerates wound epithelialization and promotes angiogenesis in proliferative phase. The role of TNF- α in angiogenesis has been either proangiogenic or antiangiogenic (55). *In vivo*, TNF-α is an activator of angiogenesis when used at low dose (56, 57) but it has been associated with inhibition of normal wound healing when found in high levels (3, 34). It is able to compensate for the negative effect of macrophage reduction on wound healing process (54). *In vitro*, TNF-α is a cytotoxic and has an inhibitory effect on endothelial cell growth (56). In the role of epithelialization activity, it stimulates keratinocyte activation and activates epithelial proliferation and migration in the closure of wound (3, 34).

2.9.3 Vascular endothelial growth factors (VEGFs)

VEGFs are families of growth factors comprising of VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placenta growth factor (58). They are produced from endothelial cells, keratinocytes, fibroblasts, platelets and macrophages. VEGFs have an important role in the wound healing process because they associate with vasculogenesis at the beginning of wound repair process. They also stimulate epithelialization, collagen deposition, migration and stimulus mounting numbers of endothelial cells. VEGFs are

secreted by platelets in the first phase of the wound healing process while macrophages release VEGFs and TNF-α. VEGFs are also produced by keratinocytes and fibroblasts (59).

At present, people worldwide show high interests in bringing natural products and advance them for natural medicines. Based on the evidence support from research findings, there are some medicinal herbs are successfully become Thai traditional medicines, including *Aloe Vera* and *Centella asiatica* (12). Rang Jued is a native plant mostly found in Thailand and neighborhoods. It is common used as an antidote for several poisonous agents including toxic plants and chemicals. Moreover from the previous pharmacological study, it was found that *T. laurifolia* possessed antitoxic, anti-inflammatory, antioxidant as well as anti-diabetic activities (15, 16). Nevertheless, there is no scientific support of its efficacy in wound healing. Therefore, it is a reason to elucidate its wound healing property in this study.

2.10 *Thunbergia laurifolia* Lindl. (TLL)

Scientific Name: *Thunbergia laurifolia* Lindl.

Family: ACANTHACEAE

Common Name: Blue trumpet vine, purple allamanda, laurel clock vine

Local Name: Rang Jued, Kumlung Changpueak, Kobchanang, Kreakaokiew, Yakiew (Central region), Rangyen (Yala), Duwao (Pattani), Tidpud (Nakon Sithammarat), Namnong (Saraburi), Yamyae, Ad-air (Petchaboon) (60).

Native Status: India (61).

Figure 8. *Thunbergia laurifolia* tree and its flower

2.10.1 Chemical structure of *T. laurifolia*

T. laurifolia contains several compounds depending on the methods of extraction as shown in table 3.

Table 3. Chemical compounds found in *T. laurifolia* extracts

No	Part & Solvent	Chemical compound	Reference
1	Aqeous leaf extract	Phenolics acids (caffeic acid and apigenin)	Oonsivilai et al., 2007 (62)
2	Ethanolic leaf extract (pigment content)	Chlorophyll derivatives (Chlorophyll a, Chlorophyll b)	Oonsivilai et al., 2007, Pongsathorn et al., 2012 (62, 63)
3	Ethanolic extract: purification of DPPH scavenging fraction	Rosmarinic acid (active compound)	Boonyarikpunchai et al., 2014 (64)
4	Methanolic aerial part extract	-Two iridoid glucosides: 8-epi-grandifloric acid and 30-O-b-glucopyranosyl-stilbericoside -seven known compounds: benzyl b-glucopyranoside, benzyl b-(20-O-b-glucopyranosyl) glucopyranoside, grandifloric acid, (E)-2-hexenyl b-glucopyranoside, hexanol b-glucopyranoside, 6-C-glucopyranosylapigenin and 6, 8-di-C-glucopyranosylapigenin	Kanchanapoom et al., 2002 (65)

In Thai traditional medicine, Rang Jued is used to treat a number of pathological conditions. The aqueous extracts of fresh leaves, dried leaves, dried root and bark are used as an antidote for insecticide, ethyl alcohol, arsenic, and strychnine poisoning. It is also used to treat fever and insect toxin poisoning (66-68). At present *T. laurifolia* is in the list of herbal medicinal products of the National List of Essential Medicines (2nd edition) B.E. 2554 (2011) as a traditional remedy for the treatment of fever and intoxication.

In Malaysia, juice from the crushed leaves of *T. laurifolia* is taken for dysmenorrhea. It is also traditional used as a poultice for wound and burn wound (14).

2.10.2 Pharmacological effects

Central nervous system (CNS) activity

Thongsaard et al. (2005) found that the methanol extract of the leaves from *T. laurifolia* 200 mg/kg (IP) could significantly increase neuronal activity in a specific brain regions responsible for reward and locomotor behavior by increasing signal intensity in various brain areas such as nucleus accumbens, globus pallidus, amygdala, frontal cortex, caudate pulamen and the hippocampus (69).

Liver detoxification and protective activity

Pramyothin et al. (2005) demonstrated the hepatoprotective activity of T. *laurifolia* in *in vitro* and *in vivo* studies. Rat hepatocytes treated with *T. laurifolia* 2.5, 5, 7.5, and 10 mg/ml were able to increase MTT reduction assay compared with ethanol alone. *In vivo* study showed that rats treated with *T. laurifolia* 25 mg/kg, (PO) for 14 days reduced serum ALT, AST and hepatic triglyceride (HTg) in comparison with ethanol control group (15).

Palipoch et al. (2011) reported that supplemented *T. laurifolia* leaf extract 0.2 mg/kg for 28 days was able to reduce lead in liver and muscle of normal fish

(*Orcochromis niloticus*) treated with 45 ppm water-borne lead (II) nitrate compared to untreated group. It also reduced oxidative stress by increase the activity of liver intrinsic antioxidants including catalase, glutathione reductase, glutathione peroxidase (70).

Antidotal activity

Tejasen and Thongthapp (1979) reported that *T. laurifolia* extract 2 ml/100 mg/BW (PO) could significantly reduce lethal rate in rats treated with folidol 20 µl/kg/BW from 56.6% to 16.6%. When T. *laurifolia* was used together with atropine, the lethality of folidol exposure significantly decreased from 56.67% to 5% (71).

Antioxidant activity

Chan and Linn (2006) reported that the tea from *T. laurifolia* leaves showed higher total phenolic content and antioxidant activity compared with the commercial herbal tea (green tea and black tea) (13).

Antibacterial activity

The methanol extract from *T. laurifolia* inhibited the growth of several bacterial strains including *P. aeruginosa* (MIC = 10mg/ml), *K. pneumoniae, S. marcescens, and P. mirabilis* (40mg/ml). The least antimicrobial activity was found in *E. coli* ATCC25913 and *A. baumannii* (MIC = 80 mg/ml). Its antibacterial activity was less than the activity of ampicillin and tetracycline (72).

Anti-inflammatory and antinociceptive activity

Charumaee et al. (1998) found that the aqueous extract of *T. laurifolia* 150 mg/kg (PO) possessed anti-inflammatory activity against carrageenan-induced paw edema in mice (73).

Wonkchalee et al. (2012) reported anti-inflammatory activity of *T. laurifolia* in hamsters which were induced liver and kidney inflammation with *Opisthorchis viverrini* and *N*-nitrosodimethylamine (NDMA). Fresh and dried *T. laurifolia* solution 100 mg/kg/day for 30 days significantly decreased ALT, ALP, BUN and creatinine. Liver size was also reduced compared to the control group (74).

Mahakunakorn et al. (2010) reported that ethanol extract of *T. laurifolia* leaf with the concentration at 400 and 1,000 mg/kg (SC) possessed analgesic and anti-inflammatory activities by inhibition of writhing test, formalin test in mice. It also inhibited NO, PGE_2, TNF-α release in LPS-stimulated RAW 264.7cells at the concentration of 6.25, 12.5 and 25 µg/ml (75).

Boonyarikpunchai et al. (2014) reported that rosmarinic acid (RA) isolated from the ethanolic extract of *T. laurifolia* leaves possessed antinociceptive activity in mice using hot-plate, acetic acid-induced writhing, and formalin tests at doses of 50 and 100 mg/kg. The anti-inflammatory activity were determined in two mouse models of carrageenan-induced paw edema and cotton pellet-induced granuloma formation, it was found that RA at 100 mg/kg significantly suppressed carrageenan-induced paw edema and inhibited cotton pellet-induced granuloma formation in mice (64).

Antidiabetic activity

Aritajat et al. (2004) treated diabetes rats with *T. laurifolia* aqueous extract 60 mg/kg/day for 15 days, the level of blood glucose was significantly decreased compared to the control group (16).

Toxicity test

Visitpongpat et al. (2003) performed the acute toxicity test of the aqueous extract of *T. laurifolia* at 10 g/kg/BW. The result revealed that there were no rats died or changing general behavior as well as the features of the visceral organs after treatment. In addition, 500 mg/kg/BW of the extract which was equivalent to the drinking amount of tea in human for twenty-eight days did not cause animal death and there was no abnormality of blood chemistry or internal organs (76).

2.11 Supercritical fluid extraction (SFE) technology

The supercritical fluid extraction (SFE) technology has advanced huge industry and a method of choice in many food processing industries. It has been well received as a clean and environmentally friendly and an alternative to organic solvent-based extraction of natural products (77). SFE extract is high value compounds from food and natural products, as well as drug delivery systems and the development of new separation techniques, such as using supercritical fluids to separate components of the extract, resulting in augmented quality and purity (78).

SFE are typically sterilized, contamination free and the valuable components remain in chemically natural state (79, 80). There are many substances used as supercritical solvents and their critical temperature and pressure such as carbon dioxide, ethane, methane, ethylene, etc. that are influenced by pressure and temperature. Supercritical carbon dioxide (SC-CO_2) is intermediates between liquid and gases and considered important in the separation processes based on the

physicochemical characteristics including density, viscosity, diffusivity and dielectric constant which are easily manipulated by pressure and temperature (81, 82).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials

3.1.1 Chemicals

- Silver sulfadiazine (Sigma, USA)
- Tri-sodium citrate (Sigma, USA)
- Sulfuric acid (Merck, Germany)
- Vanillin (Fluka, USA)
- Ethanol (Merck, Germany)
- Benzene (Merck, Germany)
- Ethyl acetate (Merck, Germany)
- Pepsin (Sigma, USA)
- Anisaldehyde (Merck, Germany)
- VEGF Antibody (Gene Tex, Netherland)
- TNF-α Antibody (Gene Tex, Netherland)
- COX-2 Antibody (Gene Tex, Netherland)
- Polyclonal Goat Antibody (DakoCytomation, Denmark)
- Sircol Assay Kit for collagen determination (Biocolor, UK)
- Xylene (Merck, Germany)
- Formaline (Vidyaom Co., Thailand)
- Skim milk (Sigma, USA)
- Biebrich scarlet (Sigma, USA)
- Acid fuchsin (Sigma, USA)
- Glacial acetic acid (Sigma, USA)
- Phosphomolybdic acid (Sigma, USA)

- Phosphotungstic acid (Sigma, USA)
- Glacial acetic acid (Sigma, USA)
- Hematoxylin stain (Sigma, USA)
- Eoscin stain (Sigma, USA)
- Carbopol® 940 (Lubrizol, USA)
- Propylene glycol (Lubrizol, USA)
- Methyl paraben (Lubrizol, USA)
- Propyl paraben (Lubrizol, USA)
- Ethylene diamine tetraacetic acid (EDTA) (Lubrizol, USA)
- Triethanolamine (Lubrizol, USA)

3.1.2 Instruments

- Autopipette (Gilson, USA)
- Electrical metal rod
- Thin Layer Chromatography set (Camag Linomat 5, Muttenz, Switzerland)
- TLC silica gel 60F 254 (Merck, Germany)
- Microplate reader (Lab system smultiskan, USA)
- Camera (Panasonic model DMC-FH7, UK)
- Centrifuge (Hettich, USA)
- Microcentrifuge tube (Hettich, Germany)
- 96-well plate (Eppendorf, USA)
- Vortex mixer (Scientific industries, USA)
- Supercritical fluid extraction CO_2 equipment (24L-SFE, China)
- Biopsy punch (Stefel, Ireland)
- Others: cotton, syringe, scissor

3.1.3 Preparation of plant

The *T. laurifolia* was collected from Klong Hat, Sra Kaew Province, Thailand in July 2012. It was identified and authenticated by Dr. Chanai Noysang (Thai Traditional Medicine College, Rajamangala University of Technology Thanyaburi, Pathumtani, Thailand) and collected in the Royal Forestry Department, Bangkok, Thailand (BKF. No183890).

Supercritical Carbon dioxide extraction

The leaves of *T. laurifolia* were washed and dried at 40ºC for 6 - 8 hrs. then 5,000 g dry leaves was extracted by supercritical fluid extraction apparatus.

Figure 9. Supercritical fluid extraction apparatus (Model: 24L-SFE, China).

5,000 g dry leaves of *T. laurifolia*

↓

Extraction: liquid CO_2 withdraw from a gas cylinder is mixed with the appropriate modifier and is introduced at a specific pressure and temperature in extraction (Pressure 20.5 Mpa: T.60°C, 8 Mpa: T. 60°C, 5 Mpa: T. 31°C) for 3 hr.

↓

The SC-CO_2 of *T. laurifolia* extract was homogeneous dark green color.

The percentage of yield was 2.38% and it was kept at - 20°C throughout the experiment.

Figure 10. Supercritical CO2 extract of TLL

3.2 Experimental animal

The experiment protocol was approved by Faculty of Medicine, Chulalongkorn University Animal Care and Use Committee (Approval No.05/56). One hundred and fifty male Wistar rats weighing 200 - 250 g were obtained from the National Laboratory Animal Center, Mahidol University, Salaya, Nakorn Pathom. The rats were housed in the air-conditioned room maintained temperature at 25 ± 1°C. They were provided with normal food and water *ad libitum* and acclimatized for 1 week before experimentation.

3.3 Determination of TLL extract fingerprints by high performance thin layer chromatography (HPTLC).

1 mg of *T. laurifolia* carbon dioxide extract was dissolved in 1 ml methanol and then filtered through a nylon membrane filter 0.2 µm, 47 mms. The methanol solution was applied into the HPTLC system as the following procedures (83):

SC-CO_2 of *T. laurifolia* leaf extract in methanol solution

↓

Injected into TLC aluminum plate and used benzene: ethyl acetate (8:2) V/V as the mobile phase for 30 minutes (Figure 11A and B)

↓

TLC plate was developed to 8 cm distance above the position of the sample application, then removed from the chamber and air dried at room temperature

↓

Measured absorbance under UV at 254 and 366 nm by TLC visualizer (Figure 11 C)

↓

Sprayed with vanillin – sulfuric acid reagent and

heating at 110°C for 10 minutes

↓

Scanned the derivertized plate immediately using Camag TLC Scanner III (Figure11 D)

and used Wincats, integrated Software 4.02, for detection of data

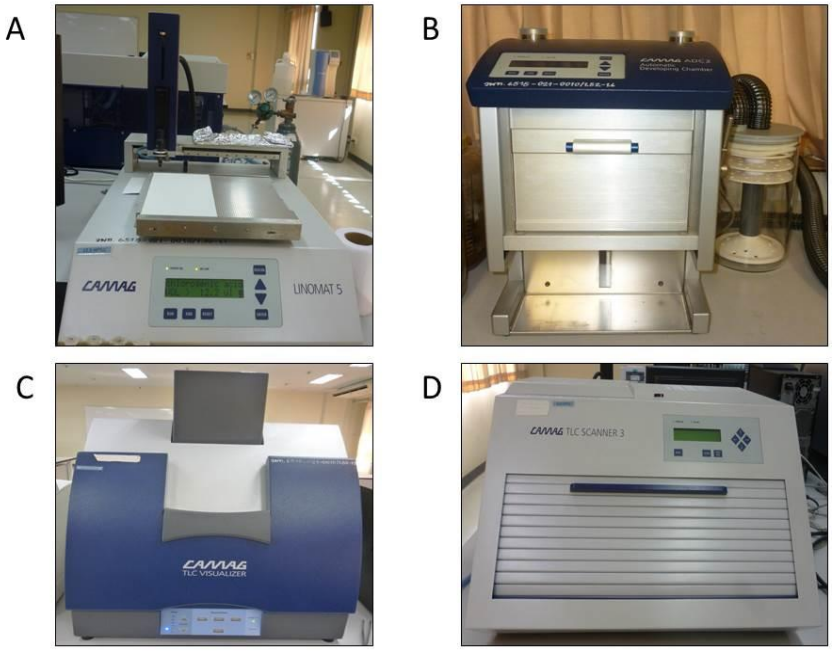


Figure 11. HPTLC equipment sets (A) HPTLC LINOMAT 5, (B) Automatic delveloping chamber, (C) TLC visualizer, (D) TLC Scanner III

3.4 Formulation of SC-CO_2 TLL extract topical gel

SC-CO_2 TLL extract topical gel was prepared by Dr. Chanai Noysang (Thai Traditional Medicine College, Rajamangala University of Technology Thanyaburi, Pathumtani, Thailand). Gel base was prepared using ingredients shown in Table 4 and incorporation with the active substances including 1% silver sulfadiazine, 2.5%, 5%, and 10% TLL extract. The prepared gel was filled in the collapsible tubes and stored at a cool dry place.

Table 4. The gel base formulation (100g).

No	Materials	For 100 g
1	carbopol® 940	0.5 g
2	propylene glycol	35 g
3	methyl paraben	0.15 g
4	propyl paraben	0.3 g
5	EDTA	0.1 g
6	triethanolamine	qs
7	distilled water	63.95 g

3.5 Induction of second degree burn

The second degree burn wound in rats were induced using the method modified from Thakur et al. (2011). The animals were anesthetized with pentobarbital sodium 60 mg/kg BW intraperitoneally (IP). The back between lower the parts of the scapula of these animals were shaved and depilated. Then they were placed with 90°C electrical metal rod (2-cm diameters) on their shaved area for 10 seconds (Figure 12). The wound involved approximately 2.5% of the total body surface area of the animals. The photographs of the wounds were taken by a digital camera and the areas of wound were measured by Image pro v.6 software. The rate of wound healing was calculated by the method described by Ravindrasingh et al. (2011). After recovering from anesthesia, the animals were housed individually with free access to water and standard laboratory chow (84, 85).

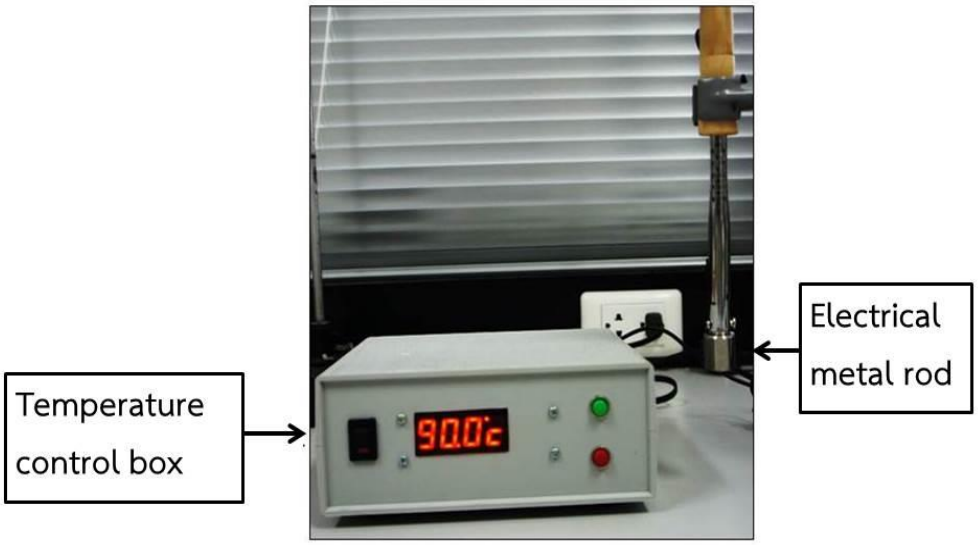


Figure 12. The electrical metal rod equipment

3.5.1 Animal preparation

A total of 150 Male Wistar rats were used in this study. They were divided into 6 groups. There were 6 rats in each group. Rats in each group received the following intervention:

1. burn (control)
2. burn treated with gel base
3. burn treated with 1% SSD gel
4. burn treated with 2.5% TLL gel
5. burn treated with 5% TLL gel
6. burn treated with 10% TLL gel

The wounds were daily treated topically with 200 mg of the test substances or gel base for 28 days except control burn group.

- On day 3, 7, 14 post burning, the animals in each group were sacrificed with pentobarbital sodium 100 mg/kg BW (IP). The isolated wound tissues were

collected and divided into 2 parts. The first part of the tissue samples were used for histopathological analysis, Masson's trichrome measurement and immunohistochemistry determination. The other part of wound tissue samples were used for collagen content analysis.

- Progressive change in wound areas were followed up every 3 days until 28 days post burning. The percentage of wound area was calculated subsequently the wound closure time was used as a parameter of wound healing.

Experimental Design

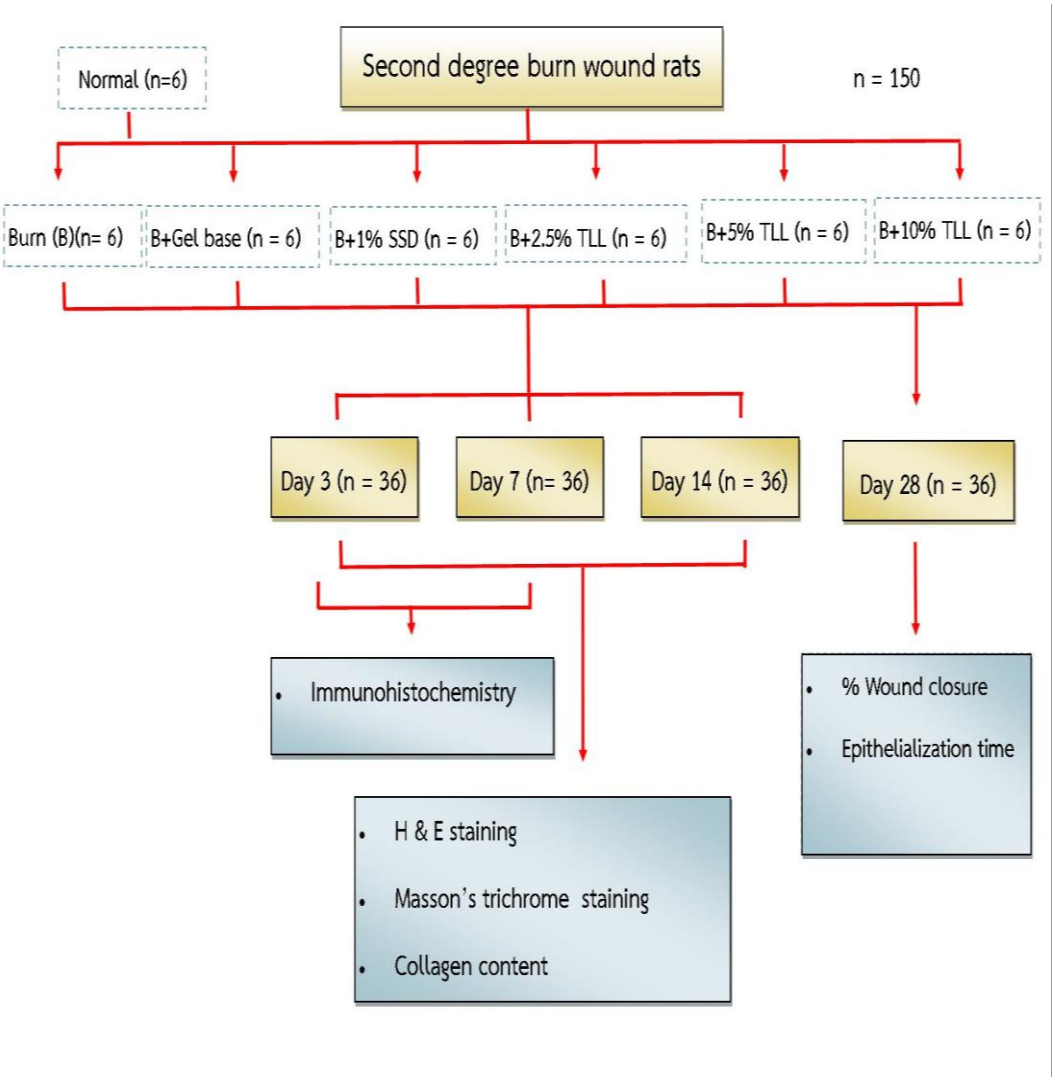


Figure 13. Diagram of experiment

3.6 Evaluation of burn wound

3.6.1 General appearance of the wound

Lesions around the wounds were examined on day 0 - 28 post burning. The wounds were examined in terms of color, exudates, swelling of wound surface and the consistency of surrounding wound tissue.

3.6.2 Rate of wound healing

The photographs of wound lesion were collect at 16 - cm distance vertically from the wound, with an automatic camera every three days, from day 0 to day 28 (Ravindrasingh et al., 2011). Subsequently, wound area was estimated by Image pro v.6 (Media Cybernetics, Inc., Rockville, MD, USA) and percentage of wound contraction was determined using the equation below and the epithelialization time was measured by the closure of the wound (85).

Percentage of wound contraction =

$$\frac{\text{Wound diameter on initial day (day 0) - Wound diameter on particular day}}{\text{Wound diameter on initial day}} \times 100$$

3.7 Collection and storage of wound tissue

The lesion of wound were cut and divided into 2 parts, the first part was fixed in 10% buffered-formalin for 24 hrs. The fixed tissues were embedded in paraffin wax and were serial dissected into 6 μm thickness for hematoxylin and eosin staining, Masson's trichome staining and immunohistochemistry study. The second part was snap frozen in liquid nitrogen and then stored at - 80°C until analysis of collagen content.

3.8 Histopathological analysis

The tissues were preserved in the fresh fixative aqueous 10% neutral for buffered formaldehyde solution for least 24 hrs. then embedded in paraffin. The section of 6 μm in thickness was cut and stained with hematoxylin and eosin dyes (Liu et al., 2008). In this study, the scores were evaluated by Assist. Prof. Waraphan Toniti, Faculty Veterinary Science and Dr. Achiraya Tammasakchai, Department of Pathobiology, Faculty of Science, Mahidol University.

3.8.1 Hematoxylin and Eosin staining

- Deparaffinized with xylene

- Dehydrated with absolute alcohol for 2 minutes (two times), 90% alcohol for 2 minutes (two times), 80% alcohol for 2 minutes (two times), 70% alcohol for 2 minutes (two times) and finally rinse in the tap water.

- Stained slide with Mayer's hematoxylin for 10 minutes and washed in the running tap water for 2 minutes.

- Stained with eosin solution for 3 minutes and shaked the slides for all the times.

- Dehydrated the slides three times for 3 minutes in each step as following: 70%, 80%, 90%, and absolute alcohol, xylene solution, and mounted.

- Light microscope with 4x and 10x objective lens was used to examine the specimen (86).

3.8.2 Masson's trichrome analysis (Masson PJ. 1929)

The specimen of skins was placed in the staining jar as the following procedures:

- Deparaffinized and hydrated with distilled water.

- Stained with Mordant in Bouin's solution for 1 hour at 56°C, overnight at room temperature.

- Cooled and washed in running water until yellow color disappeared, rinsed in distilled water.

- Stained with Weigert's iron hematoxylin solution for 10 minutes, washed in running water 10 minutes, rinsed in distilled water.

- Stained with Biebrich scarlet-acid fuchsin solution for 2 minutes.

- Rinsed in distilled water.

-Washed with phosphomolybdic-phosphotungstic acid solution for 10 - 15 minutes.

- Stained with aniline blue solution for 5 minutes.

- Rinsed in distilled water.

- Added glacial acetic solution for 3 to 5 minutes.

- Dehydrated in 95% alcohol, absolute alcohol, and clear in xylene, two changes each.

- Mounted with histoclad (87).

3.9 Immunohistochemistry analysis

The wound were cut and placed on slides, as the following procedures:

- Deparaffined and dehydrated tissue sections as described.

- Incubated tissues for 5 minutes with 3% hydrogen peroxide in distilled water and rinsing.

- Placed tissues in Tris buffered saline (TBS) for 5 minute, incubated for 20 minutes with normal rabbit serum (Dako, 447) dilute 1:5 in TBS for blocking of non- specific.

background.

- Incubated at 4°C overnight, with mouse monoclonal antibody diluted in TBS and tap off antibody.

- Incubated tissue for 30 minutes with rabbit polyclonal antibody to TNF-α, COX-2, VEGF (diluted 1:200) in TBS, tab off biotinylated antibody and placed slide in TBS for 5 minutes.

- Incubated for 30 minutes with Horseradish peroxidase (HRP), tap off and placed slide in TBS for 5 minutes.

- Incubated for 10 minutes with Diaminobenzidine (DAB) substrate, counter stained and mounted with coverslip.

3.10 Collagen content

Collagen deposition at wound area was estimated by determining the total wound collagen content using the Sircol Collagen Assay Kit (Biocolor, Northern Ireland UK) which is a quantitative dye-binding assay for measurement of the total collagen (type I-V) in tissue. The tissue biopsies were digested by pepsin (100 mg pepsin/gram wet tissue) in 0.5 ml of 0.5 M acetic acid, overnight at 4°C, then the samples were centrifuge 12,000 g, 10 minutes.

- Added 1 ml of Sircol Dye reagent in each sample, and then mixed for 30 minutes.

- Transferred the sample to micro centrifuge tube and spin at 12,000 x g, 10 minutes.

- Suspended the pellets in 1 ml of alkali reagent, mixed the samples for 10 minutes by a vortex mixer.

- Assessed collagen content colorimetrically at 540 nm using microplate reader.

- Constructed the collagen standard curve for calculation of the collagen content in the test sample.

3.11 Statistical analysis

Results were presented as mean ± standard deviation of mean (mean ± SD). The differences among the experimental groups were compared by ANOVA (analysis of variance) test and then by Tukey's post hoc test. P - Values < 0.05 were considered to be statistically significance. SPSS version 21.0 (SPSS Inc., Chicago, IL) was used for statistical analysis.

CHAPTER IV

RESULTS

4.1 Chromatographic fingerprint analysis SC-CO2 of *T. laurifolia* leaf extract by HPTLC technique.

HPTLC profiles SC-CO_2 of *T. laurifolia* leaf extract under UV 254 nm, 366 nm, Vis, and vanillin - sulfuric acid reagent, 10 min/110^0 C → vis was recorded with the HPTLC system. Figure 14 showed 8 main spots on the HPTLC plate with the Rf values in the range of 0.05 to 0.70. The HPTLC profiles and the chromatograms detected under UV 254 nm. were demonstrated in Table 5 and Figure 15.

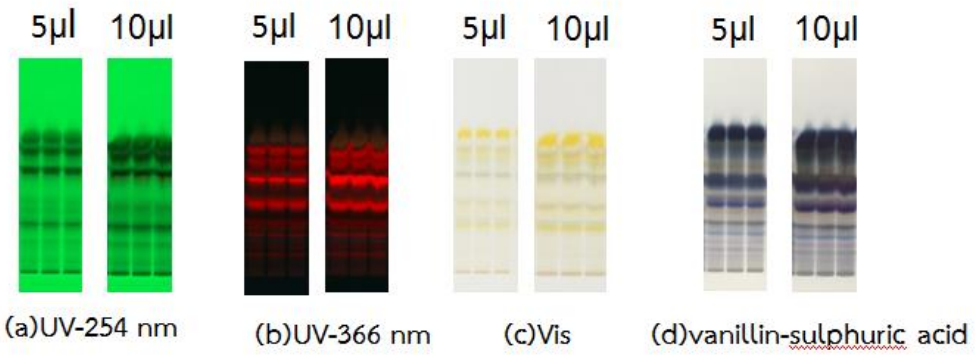
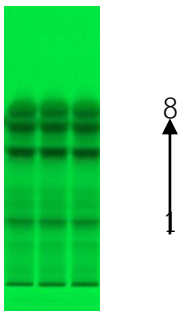
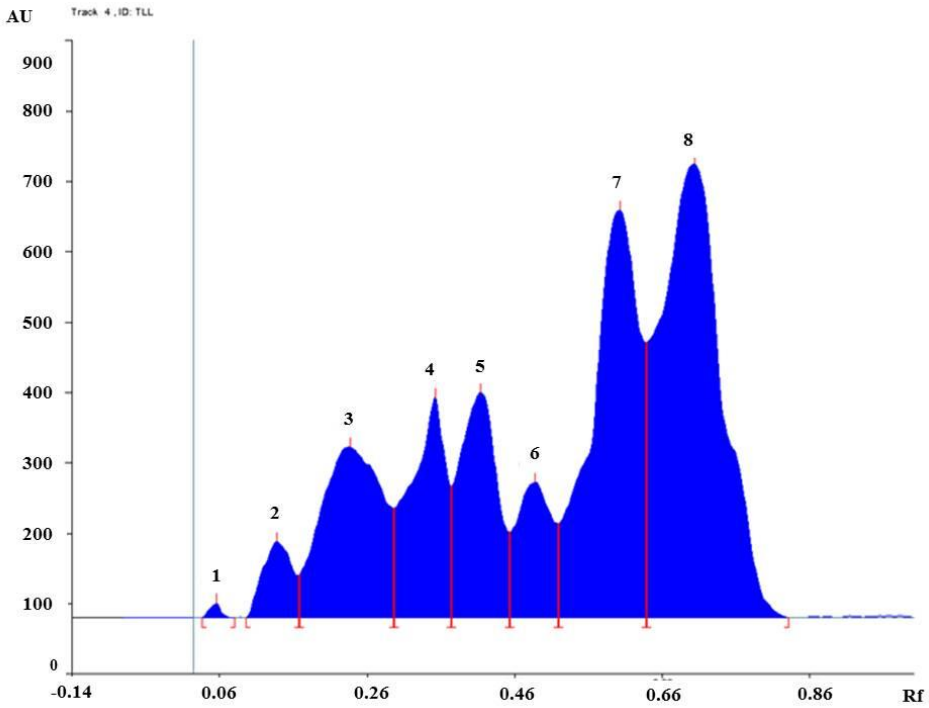


Figure 14. HPTLC - fingerprint of the SC-CO2 of *T. laurifolia* leaf extract with (a) UV-254 nm; (b) UV - 366 nm; (c) Vis; and (d) vanillin - sulfuric acid reagent.

Table 5. HPTLC profile of SC-CO2 of *T. laurifolia* leaf extract after UV-254 nm detection.

Sample	Peak	R_f	Height	Area	%Area
 **UV-254**	1	0.05	20.7	248.8	0.20
	2	0.13	107.4	3474.2	2.75
	3	0.23	242.1	16309.0	12.90
	4	0.35	312.0	12104.0	9.57
	5	0.41	319.9	13147.2	10.40
	6	0.49	192.3	7384.0	5.84
	7	0.60	578.3	30322.0	23.98
	8	0.70	644.5	43478.9	34.38



Rf =retention factor

Figure 15. HPTLC chromatogram SC-CO2 of *T. laurifolia* leaf extract with UV - 254 nm. detection showed 8 different peaks of the chemical constituents.

4.2 Effects of TLL gel on wound healing time in second degree burn wound rats.

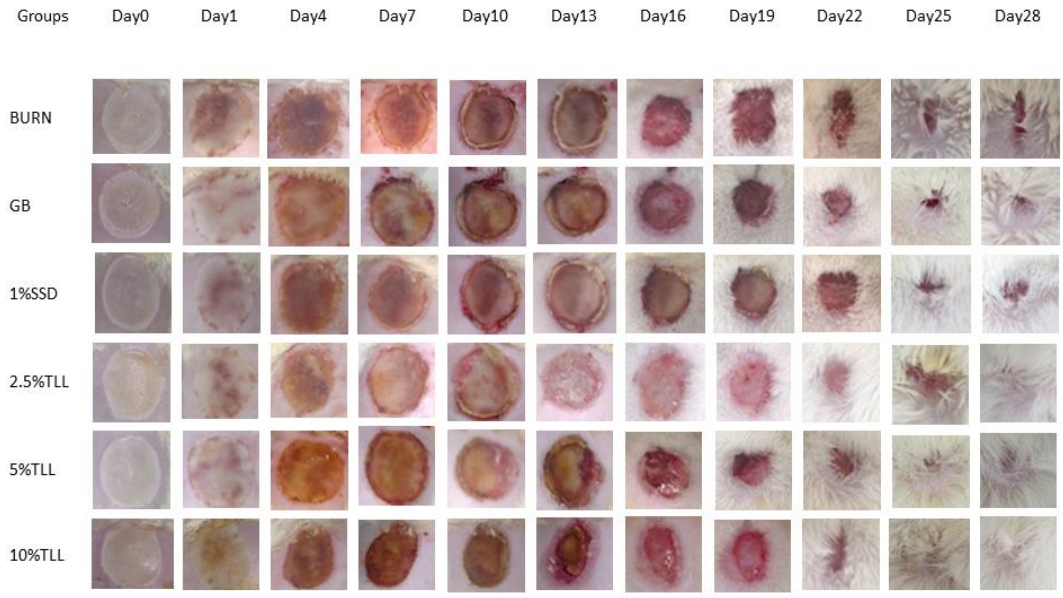


Figure 16. Effect of TLL gel on macroscopic change in wound healing patterns of control burn, gel base, 1% SSD, 2.5%, 5%, and 10% TLL gel treated second degree burn wound rats (n=6).

The lesion of wounds were examined every 3 days after burn until day 28. The macroscopic changes and the area surface of the wounds were measured as described. It was found that there was an increase in the percentage of wound closure in 10% TLL treated group than control burn group from day 4 to day 28 of treatment which was statistically significant different from burn group ($P < 0.05$) shown in figure 16, 17 and table 6. The wounds in 10% TLL gel treated group were healed completely (100%) on day 25, which was significantly more rapid than burn group (30.17 ± 0.48 day, $P < 0.01$).

For 2.5% and 5% TLL treated groups, the wounds were completely healed on day 28 post treatment in both groups ($P < 0.01$). While gel base and 1% SSD treated groups required more than 28 days for complete wound healing. The mean epithelialization times for 2.5%, 5%, and 10% TLL gel treated group were 26.83 ± 0.31, 26 ± 0.37 and 23.83 ± 0.65 days, which were faster than burn group ($P < 0.05$, $P < 0.01$) respectively. While the epithelialization time of 1 % SSD, gel base were 27.5 ± 1.20, 29 ± 0.86 days respectively which were not significant difference from burn group (Figure 18 and Table 6).

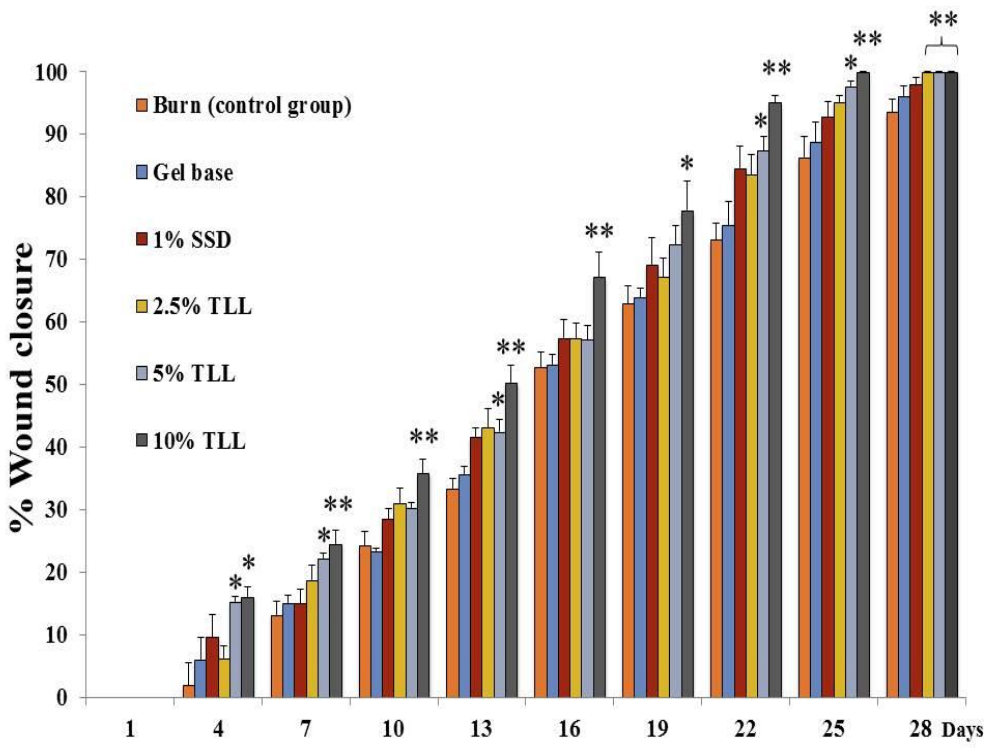


Figure 17. Effect of TLL gel on the percentage of wound closure from day 1 to day 28 post treatment in second degree burn wound rats. Data was presented as mean ± SD (n=6). Accelerating wound closure effect was demonstrated in 2.5%, 5%, and 10% TLL gel treated groups *$P < 0.05$; **$P < 0.01$ denotes significant difference from burn group.

Table 6. Effect of TLL gel in second degree burn wound rats measured as the percentage of wound closure and epithelialization

	Percentage of wound closure										
Groups	D 1	D 4	D 7	D 10	D 13	D 16	D 19	D 22	D 25	D 28	Epithelialization Time (days)
Burn	- 14.78 ± 2.97	1.84 ± 3.59	13.11 ± 2.26	24.33 ± 2.13	33.37 ± 1.55	52.72 ± 2.38	62.9 ± 2.8	73.08 ± 2.59	86.26 ± 3.28	93.61 ± 2	30.17 ± 0.48
Gel base	-15.74 ± 3.47	5.94 ± 3.67	15.03 ± 1.22	23.37 ± 0.36	35.61 ± 1.22	53.14 ± 1.72	63.94 ± 1.44	75.53 ± 3.72	88.74 ± 3.26	95.96 ± 1.67	29 ± 0.86
1% SSD	- 8.23 ± 2.29	9.55 ± 3.64	15.03 ± 2.13	28.42 ± 1.78	41.5 ± 1.59	57.45 ± 2.95	69.18 ± 4.29	84.55 ± 3.53	92.79 ± 2.31	97.9 ± 1.17	27.50 ± 1.20
2.5% TLL	-17.79 ± 3.03	6.17 ± 1.96	18.69 ± 2.48	30.99 ± 2.37	43.1* ± 3.05	57.43 ± 2.44	67.11 ± 3.03	83.53 ± 3.12	95.14 ± 0.94	100** ± 0	26.83 ± 0.31*
5% TLL	-9.57 ± 1.7	15.3* ± 0.72	22.12* ± 0.88	30.26 ± 0.88	42.42 ± 1.99	57.14 ± 2.29	72.3 ± 3.11	87.33* ± 2.37	97.66* ± 0.82	100** ± 0	26 ± 0.37**
10% TLL	-9.18 ± 2.1	16.06* ± 1.48	24.51** ± 2.14	35.76** ± 2.17	50.26** ± 2.7	67.13** ± 3.97	77.78* ± 4.66	95.05** ± 1.17	100** ± 0	100** ± 0	23.83 ± 0.65**

The data is presented as mean ± SD (n=6), * $P < 0.05$; ** $P < 0.01$ denotes significant difference from burn group.

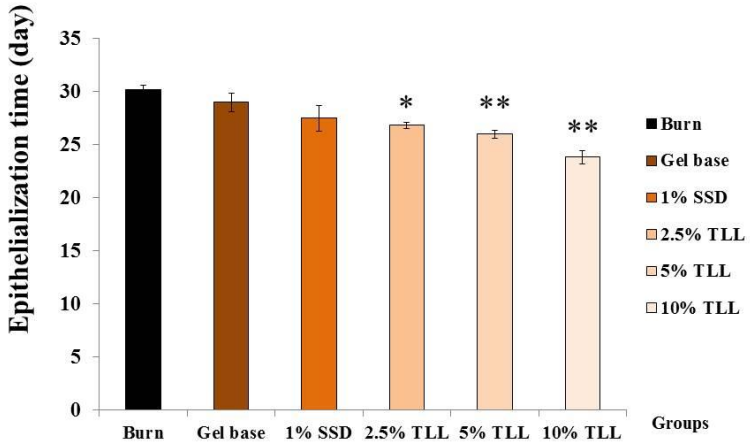


Figure 18. Effect of TLL gel on the epithelialization time in second degree burn wound rats. Data was presented as mean ± SD (n=6). Accelerating epithelialization time was demonstrated in 2.5%, 5%, and 10% TLL gel treated groups. *$P < 0.05$; **$P < 0.01$ denotes significant difference from burn group.

4.3 Effects of TLL gel in second degree burn wound rats by histopathological analysis.

Histopathological observation of wound healing was examined on day 3, 7, and 14 after burning.

On day 3 after burn operation, all groups showed damage of the dermis of the burn wound with epidermal detachment, scabs formation of necrotic tissue remnants, hyperemic vessels, no hair follicles or sebaceous glands, and phagocytic cell infiltration (Figure 19 and Table 7). The wounds of all TLL gel treated groups had no edema or congestion.

On day 7, epithelialization, fibroblast infiltration, and angiogenesis were found in all TLL gel treated groups, more than in the untreated control group. Scabbing still persisted in all groups, but scabs were thinner in all TLL gel groups. All groups had damaged epidermis and dermis with epithelialization and fibroblast infiltration. Mononuclear cell infiltration was found in all groups (Figure 20 and Table 7).

On day 14, all TLL gel treated groups showed clearly developed epithelialization, angiogenesis, and hair follicles, more than the other groups. Extensive fibroblast proliferation was observed in the 10% gel group (Figure 21 and Table 7).

Table 7. Histopathological scores on day 3, day 7, and day 14 in burn wound rats after topical application of TLL gel and control group.

Groups	Burn			Gel base			1% SSD			2.5% TLL			5% TLL			10% TLL		
	Day3	Day7	Day14	Day3	Day7	Day14	Day3	Day7	Day14	Day3	Day7	Day14	Day3	Day7	Day14	Day3	Day7	Day14
Oedema	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Congestion	++	+	-	++	-	-	+	-	-	-	-	-	-	-	-	-	-	-
Necrosis	+++	+++	++	+++	+++	++	+++	++	++	++	++	+	++	++	+	++	++	+
Infiltration of inflammatory cells	+++	+++	+++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	++	+++	+++	++
Proliferation of fibroblasts	+	++	+++	+	+++	+++	+	++	+++	+	+++	+++	+	++	+++	+	+++	++++
Blood vessel	-	-	++	-	+	++	-	+	+	-	+	++	-	+	++	-	+	++
Epithelialization	-	+	++	-	+	++	-	++	++	-	+	++	-	++	++	-	++	++
Hair follicle	-	-	+	-	-	++	-	-	++	-	-	++	-	+	++	-	+	++

+ slight; ++ moderate; +++ marked; ++++ extensive; - absent

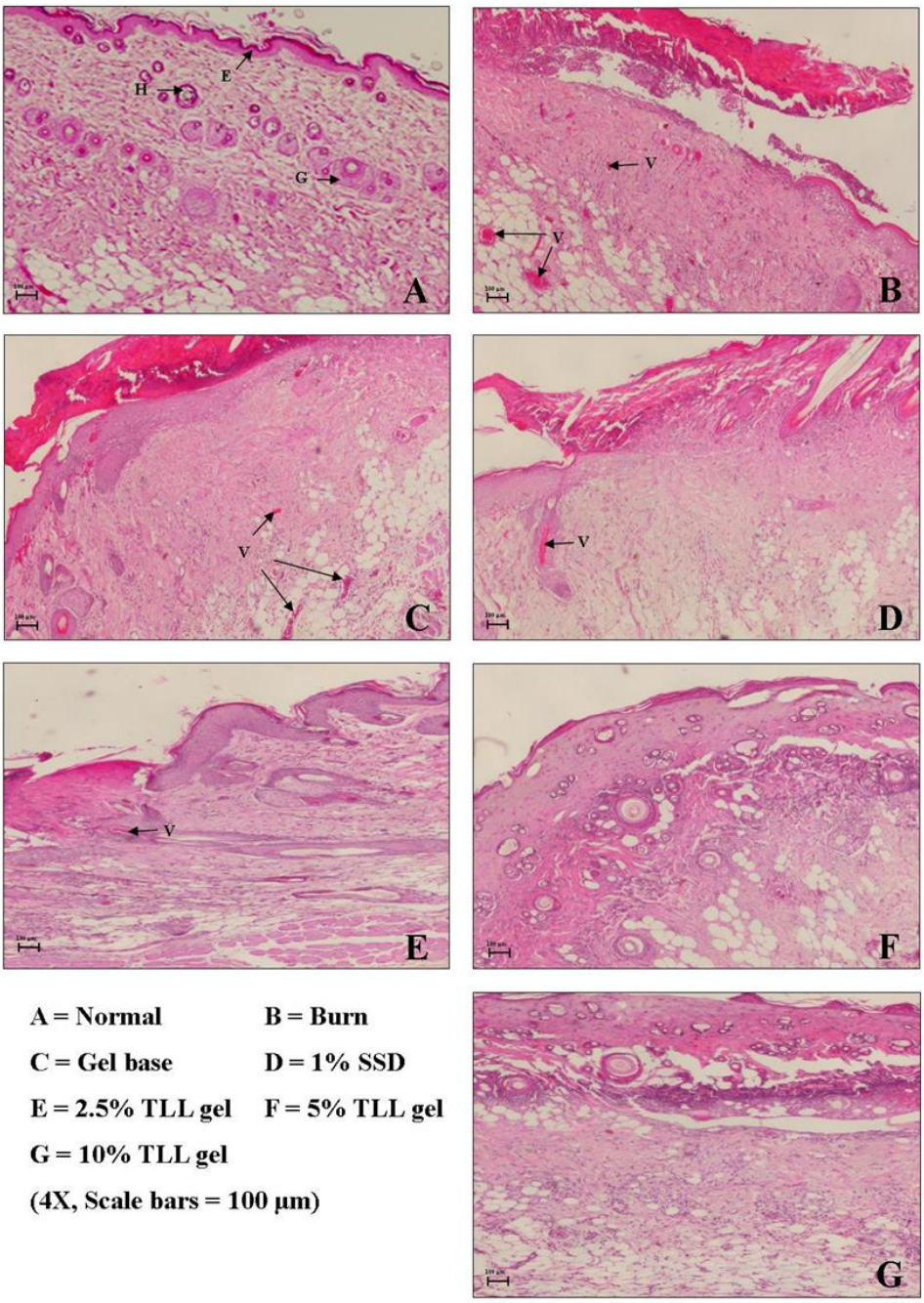


Figure 19. Histopathological analysis of skin section on day 3 post burning in second degree burn wound rats after treatment with gel base, 1% SSD, or 2.5%, 5%, and 10% TLL gel (E = epidermis, H = hair follicle, G = sebaceous gland, V = blood vessel) (n=6).

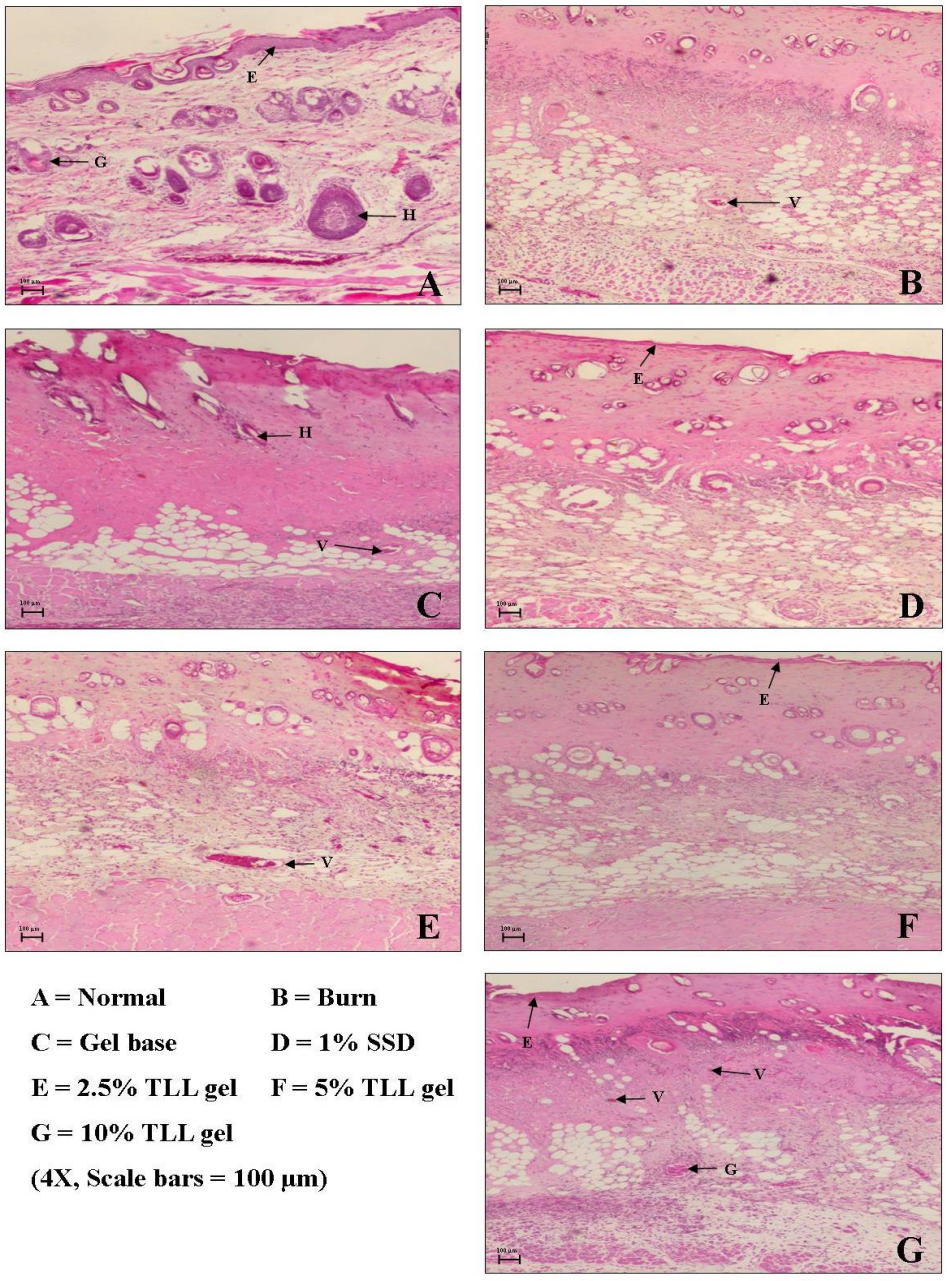


Figure 20. Histopathological analysis of skin section on day 7 post burning in second degree burn wound rats after treatment with gel base, 1% SSD, or 2.5%, 5%, and 10% TLL gel (E = epidermis, H = hair follicle, G = sebaceous gland, V = blood vessel) (n=6).

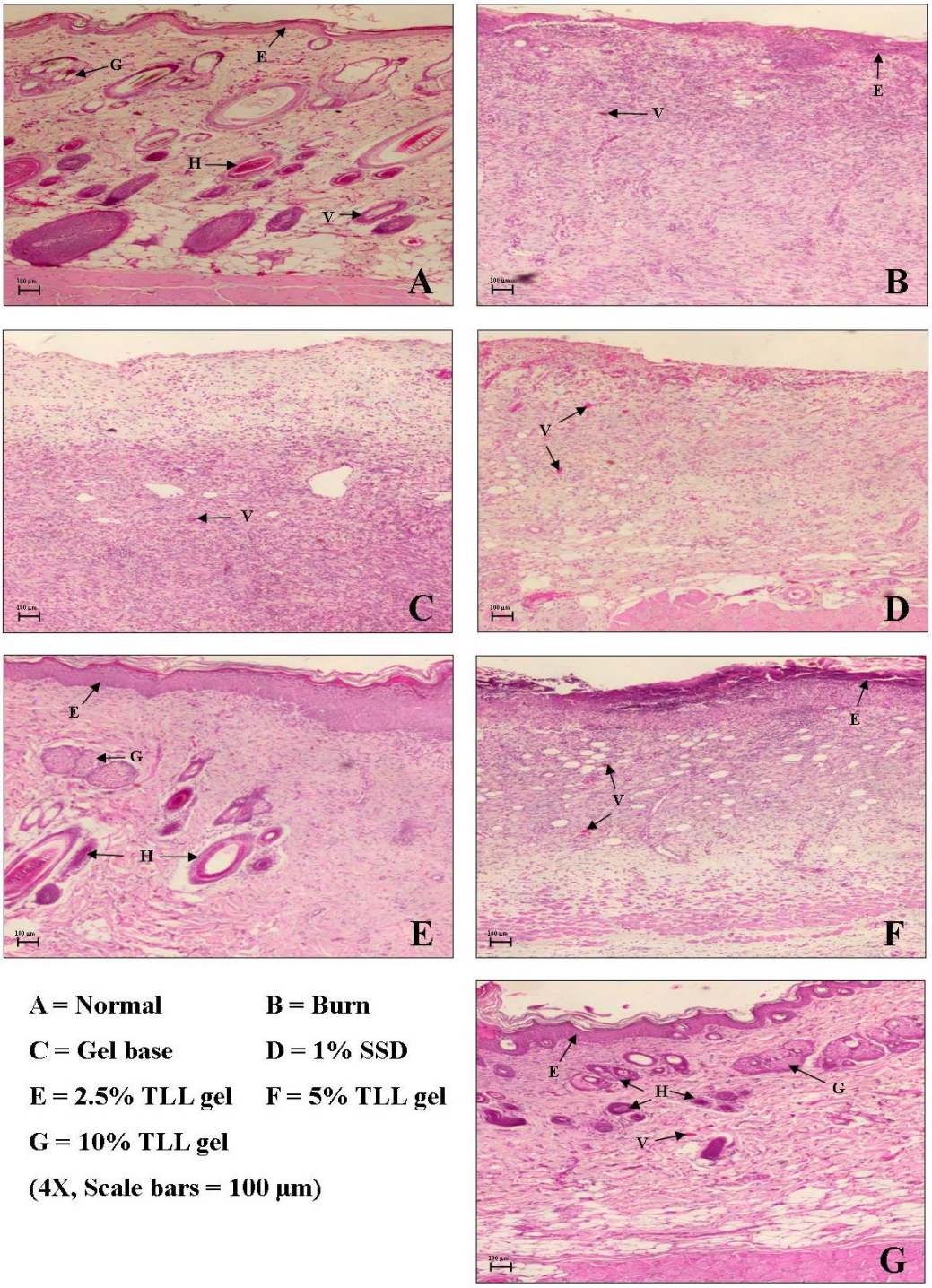


Figure 21. Histopathological analysis of skin section on day 14 post burning in second degree burn wound rats after treatment with gel base, 1% SSD, or 2.5%, 5%, and 10% TLL gel (E = epidermis, H = hair follicle, G = sebaceous gland, V = blood vessel) (n=6).

4.4 Effects of TLL gel in second degree burn wound rats by Masson's trichrome staining.

Results from Masson's trichrome staining can clearly differentiate important morphological keys for wound healing assessment. Keratin, haemoglobin, and muscle fiber are stained red color. Cytoplasm and adipose cells are stained light red or pink. Cell nuclei show dark brown to black, and collagen fiber is stained blue.

On day 3, all groups demonstrated acute inflammation of wound with clot formation, scarring, and minimal content of collagen (Figure 22).

On day 7, epithelialization was accelerated and epithelial migration was noted. All TLL gel treated groups and the 1% SSD group showed a decrease in inflammatory cells, whereas the untreated burn group still had inflammatory cells infiltration (Figure 23).

On day 14, all TLL gel treated groups, most notably the 10% TLL group, showed tissue healing with higher levels of re-epithelialization, fibroblast proliferation, and collagen fiber, as compared to the other groups (Figure 24).

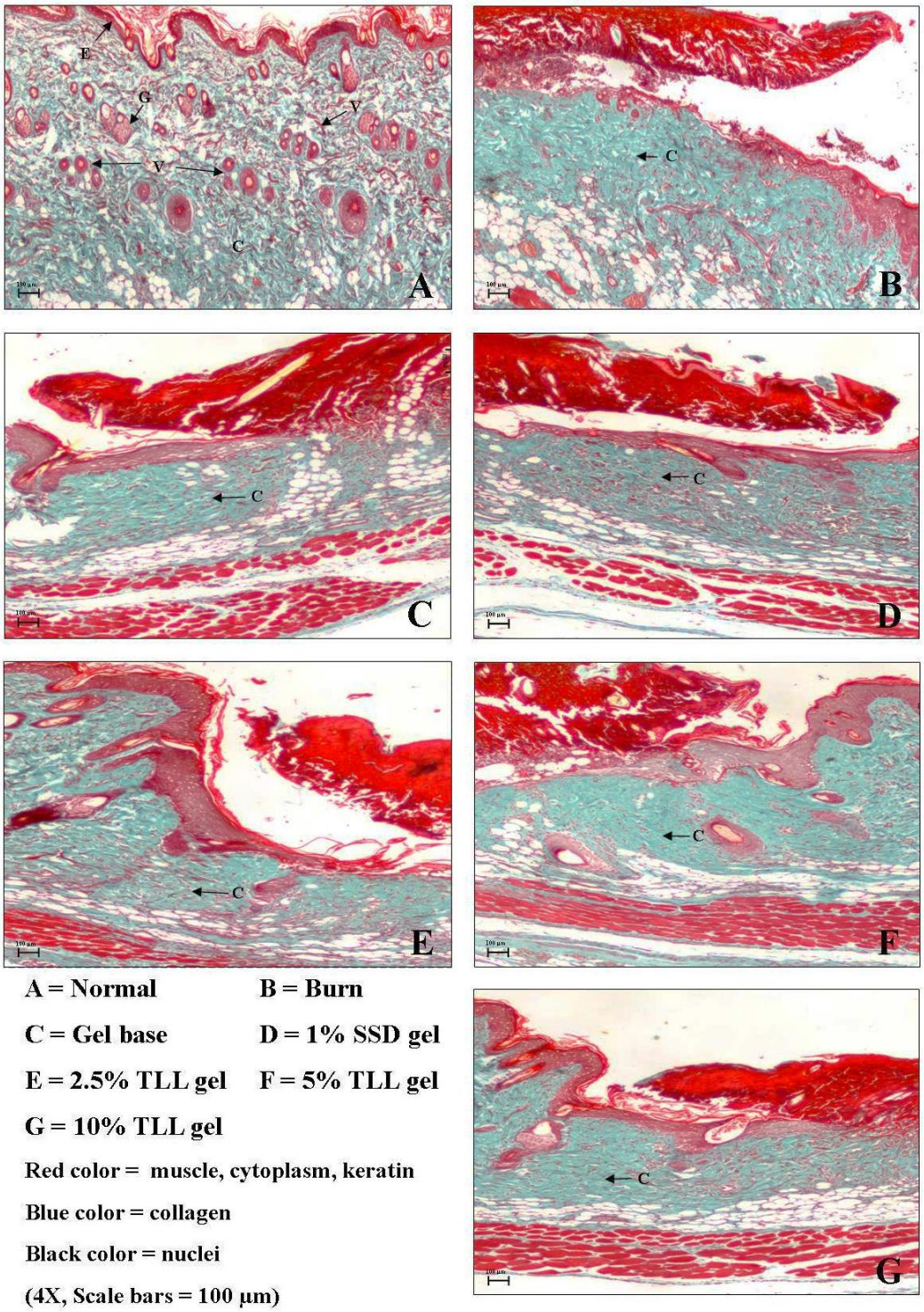


Figure 22. Masson's trichrome analysis, on day 3 post burning in second degree burn wound rats treated with gel base, 1% SSD, or 2.5%, 5%, and 10% TLL gel (E = epidermis, H = hair follicle, C = collagen, G = sebaceous gland, V = blood vessel) (n=6).

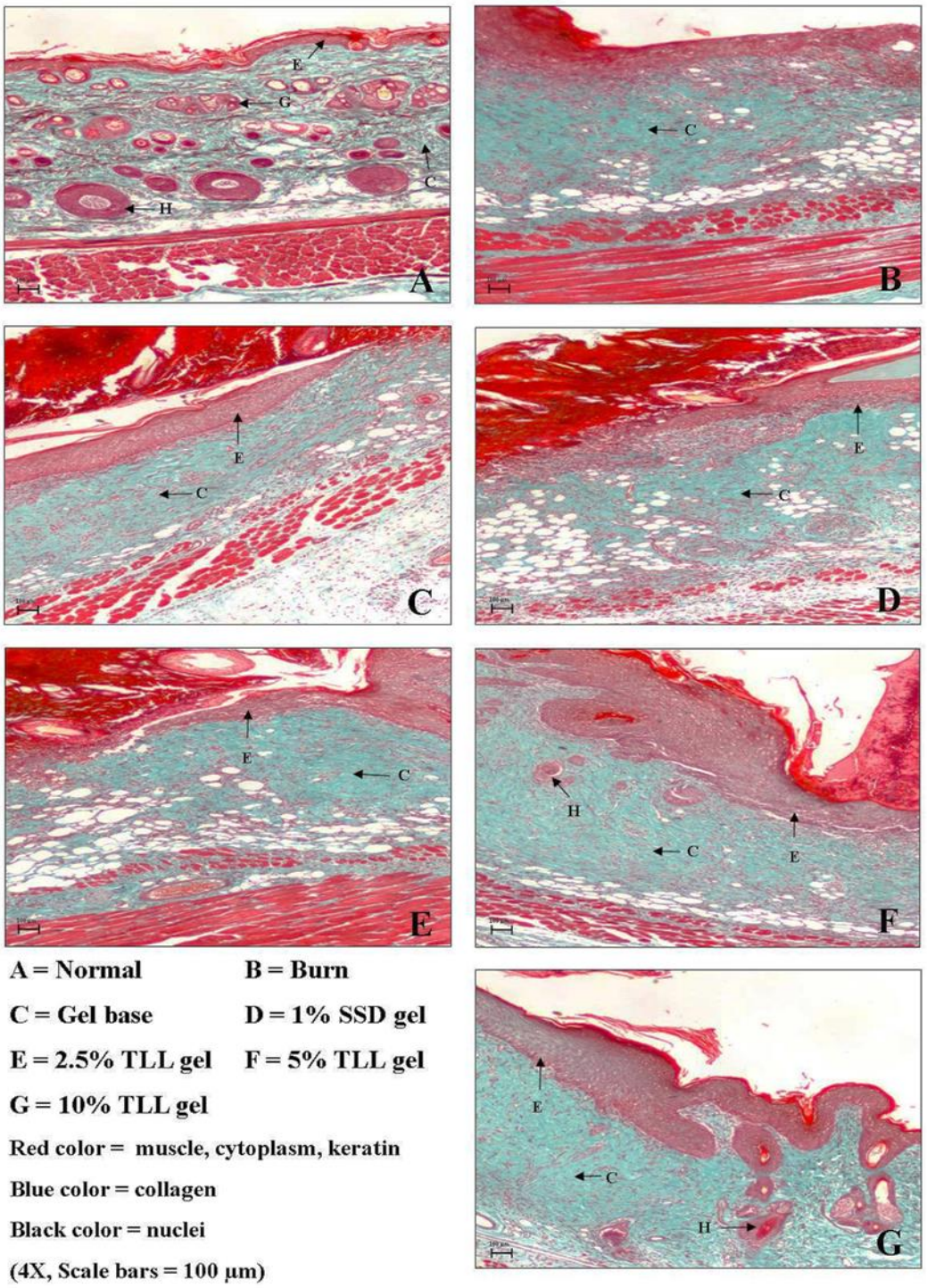


Figure 23. Masson's trichrome analysis, on day 7 post burning in second degree burn wound rats treated with gel base, 1% SSD, or 2.5%, 5%, and 10% TLL gel (E = epidermis, H = hair follicle, C = collagen, G = sebaceous gland, V = blood vessel) (n=6).

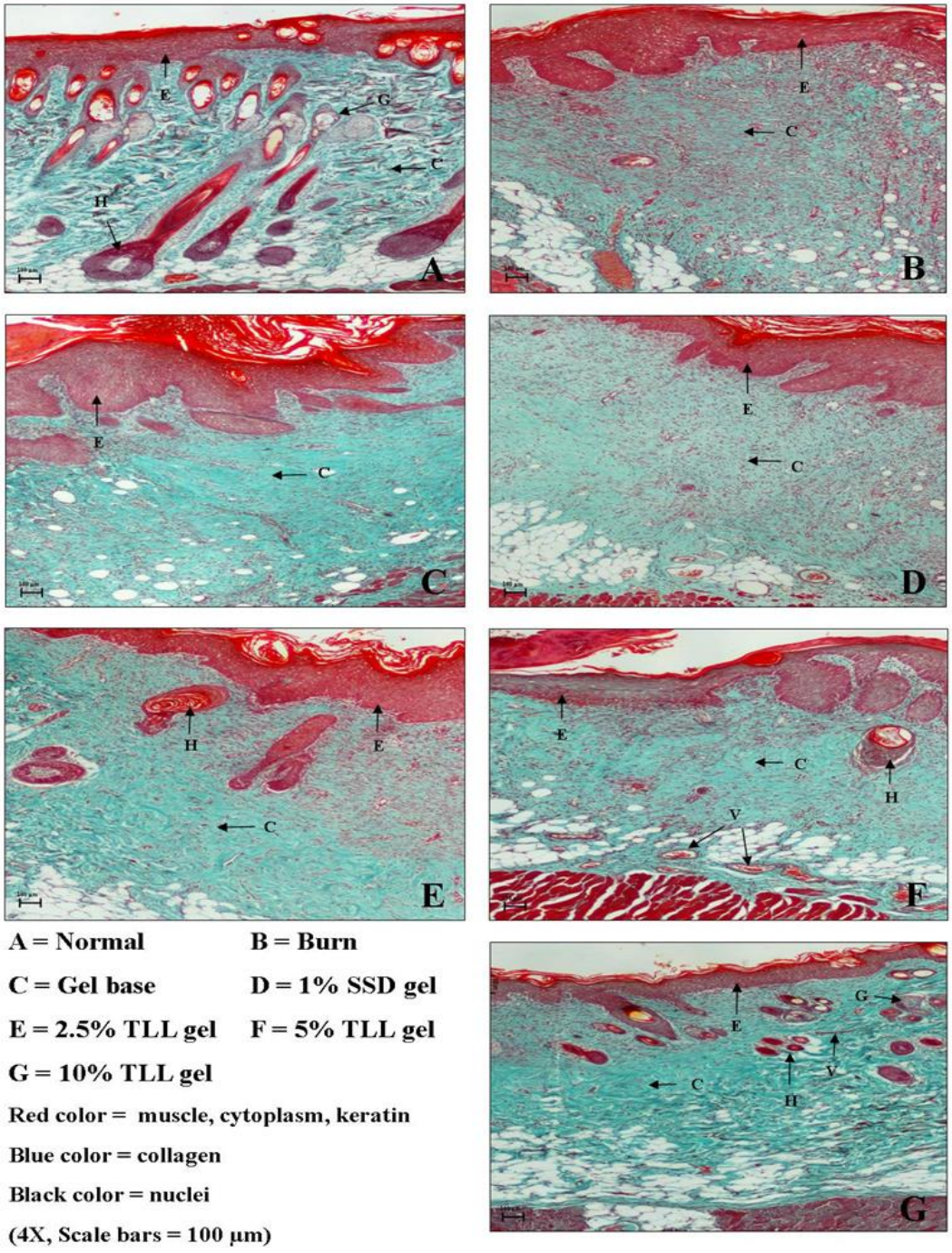


Figure 24. Masson's trichrome analysis, on day 14 post burning in second degree burn wound rats treated with gel base, 1% SSD, or 2.5 %, 5%, and 10% TLL gel (E = epidermis, H = hair follicle, C = collagen, G = sebaceous gland, V = blood vessel) (n=6).

4.5 Effects of TLL gel in second degree burn wound rats on COX-2, TNF-α, VEGF using immunohistochemistry analysis.

Immunohistochemistry refers to the process of detecting antigens cells of a tissue section by exploiting the principle of antibodies binding specifically to antigens in biological tissues and localization of biomarkers and differentially expressed proteins in different parts of a biological tissue. In this study, immunohistochemistry staining is used for determination of a pro-inflammatory cytokine: TNF-α, an inducible enzyme: COX-2, and a growth factor: VEGF in the wound tissue of rats with second degree burn on day 3 and day 7 post burning. The wound tissues stained with their specific antibodies were represented by the brown color.

Increased COX-2 expression was found in the epidermis and dermis on day 3 post burning in the control group and it was marked increased on day 7 post burning, while COX-2 expression in 10 % TLL group was found in the lesser extent on day 7 compared to control group (Figure 25, 26 and Table 8).

As the proliferative phase begins, there is an increased in the factors involved in angiogenesis including TNF-α and VEGF. It was found that TNF-α and VEGF expression were presented in all TLL treated groups both in day 3 and day 7 post burning. In contrary, with the burn group that TNF-α and VEGF were found only on day 7 post burning (Figure 27 - 30 and Table 8).

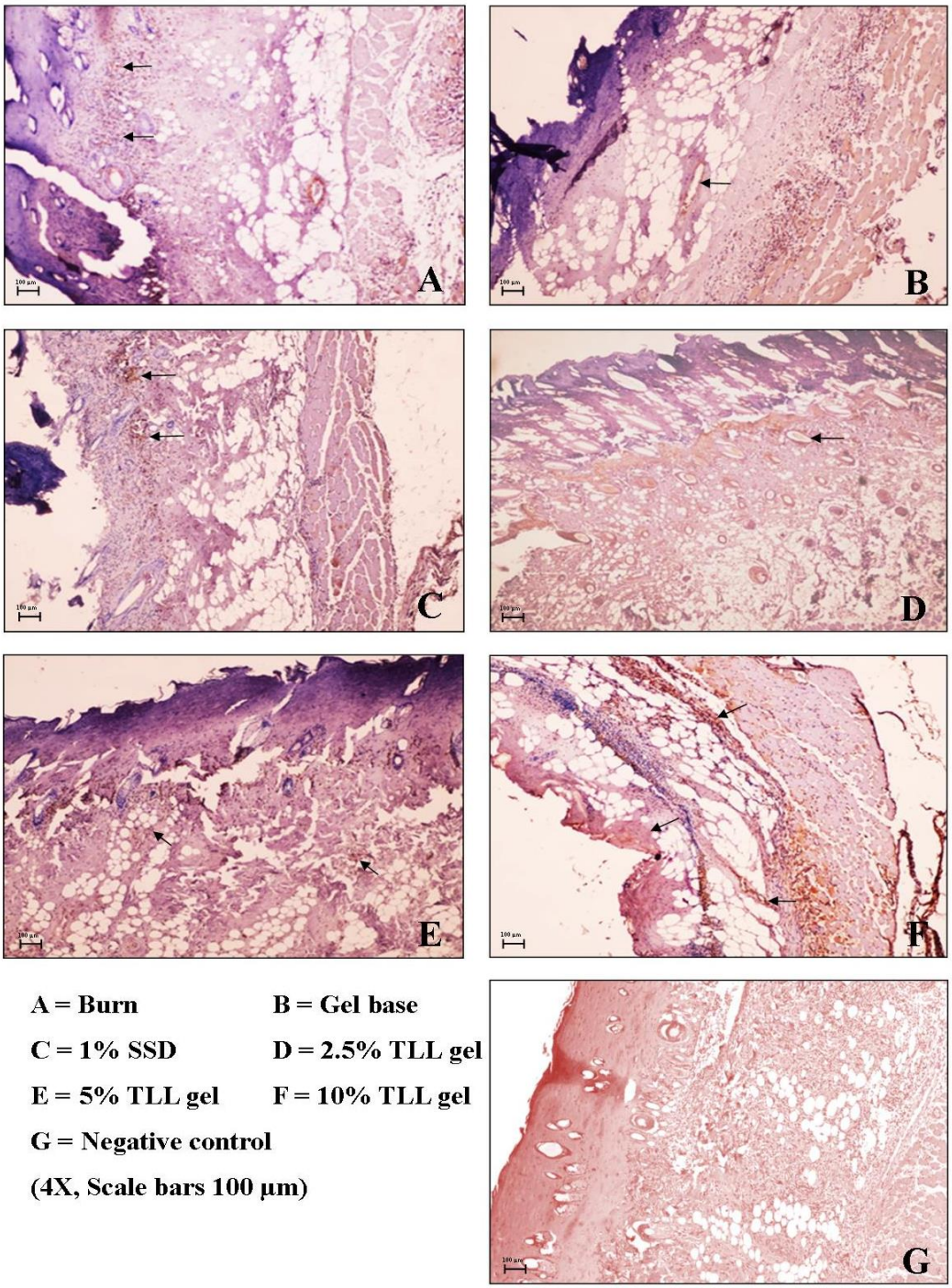


Figure 25. Immunohistochemistry analysis of skin wound. The sections were stained with monoclonal antibody for COX-2 on day 3 post burning. Arrows indicated COX-2 staining (brown color) (n=3).

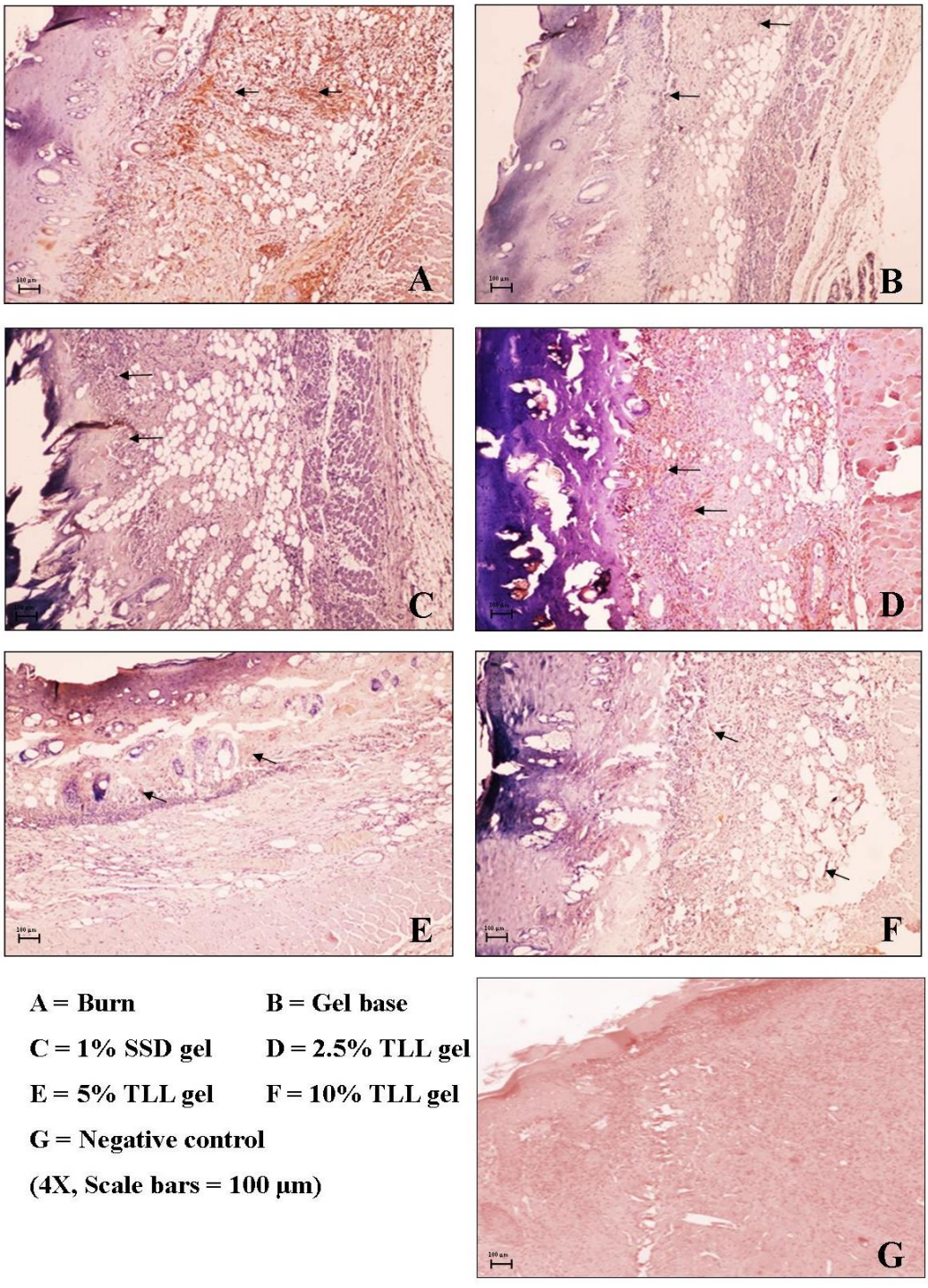


Figure 26. Immunohistochemistry analysis of skin wound. The sections were stained with monoclonal antibody for COX-2 on day 7 post burning. Arrows indicated COX-2 staining (brown color) (n=3).

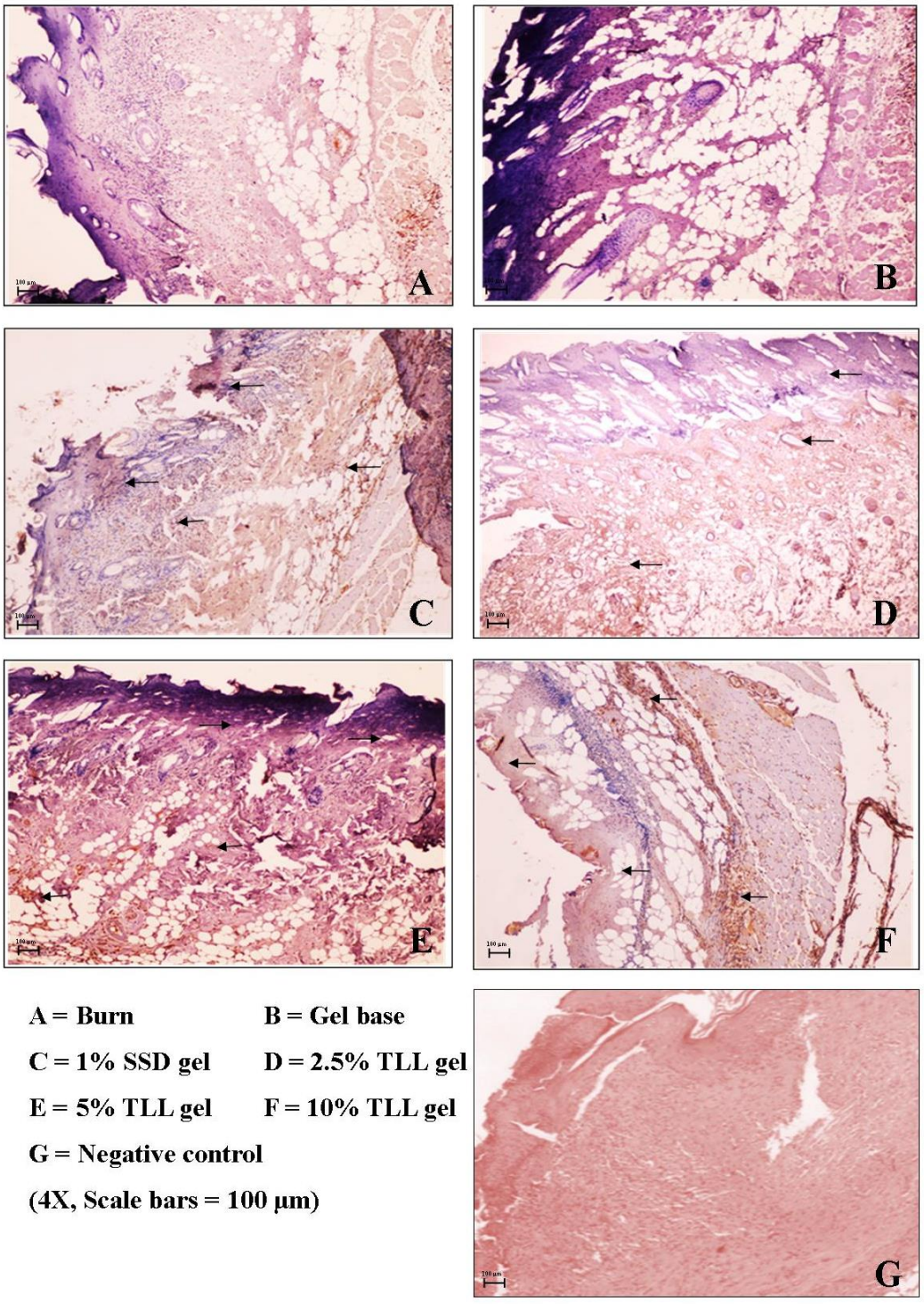


Figure 27. Immunohistochemistry analysis of skin wound. The sections were stained with monoclonal antibody for TNF-α on day 3 post burning. Arrows indicated TNF-α staining (brown color) (n=3).

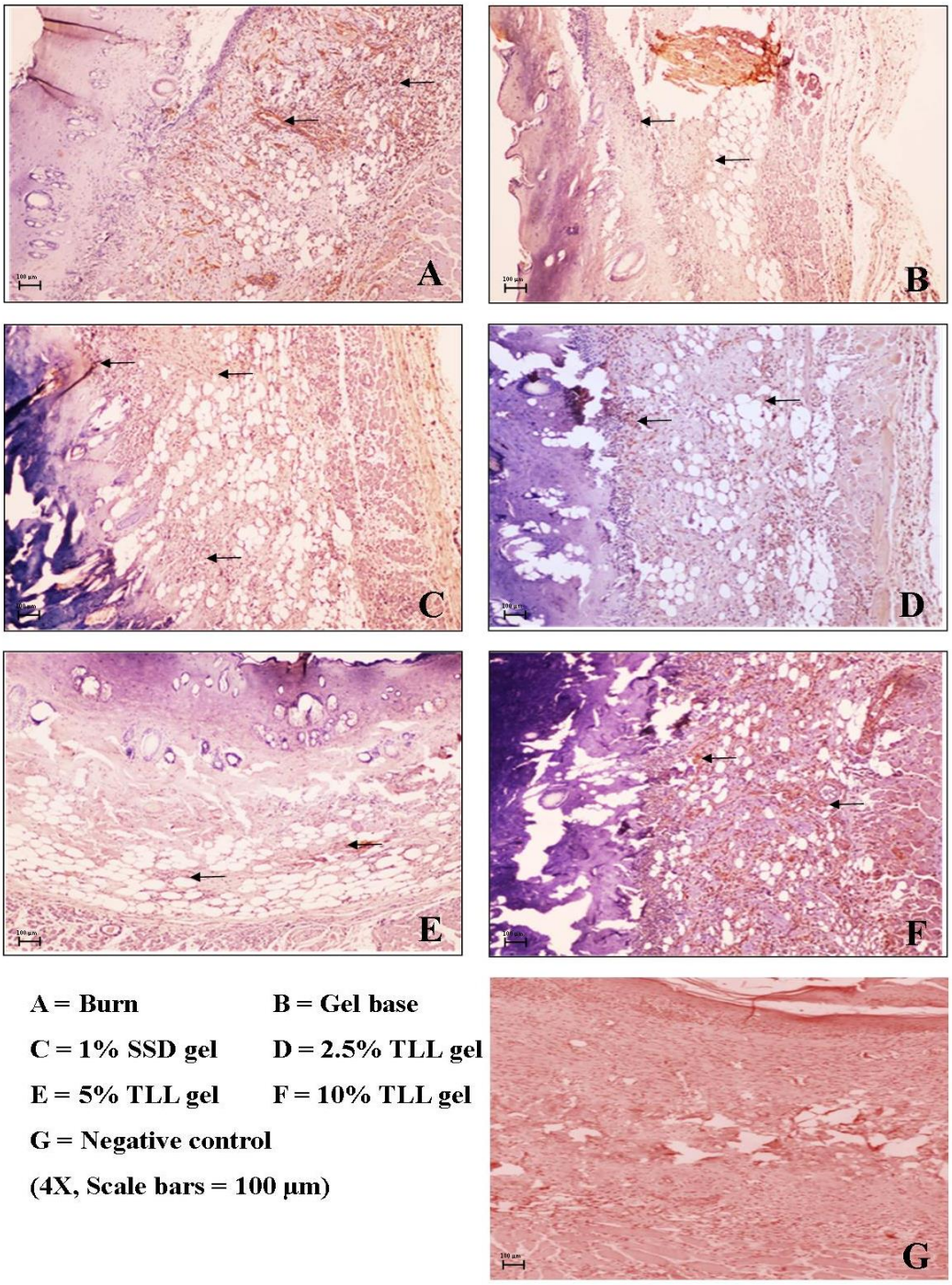


Figure 28. Immunohistochemistry analysis of skin wound. The sections were stained with monoclonal antibody for TNF-**α** on day 7 post burning. Arrows indicated TNF-**α** staining (brown color) (n=3).

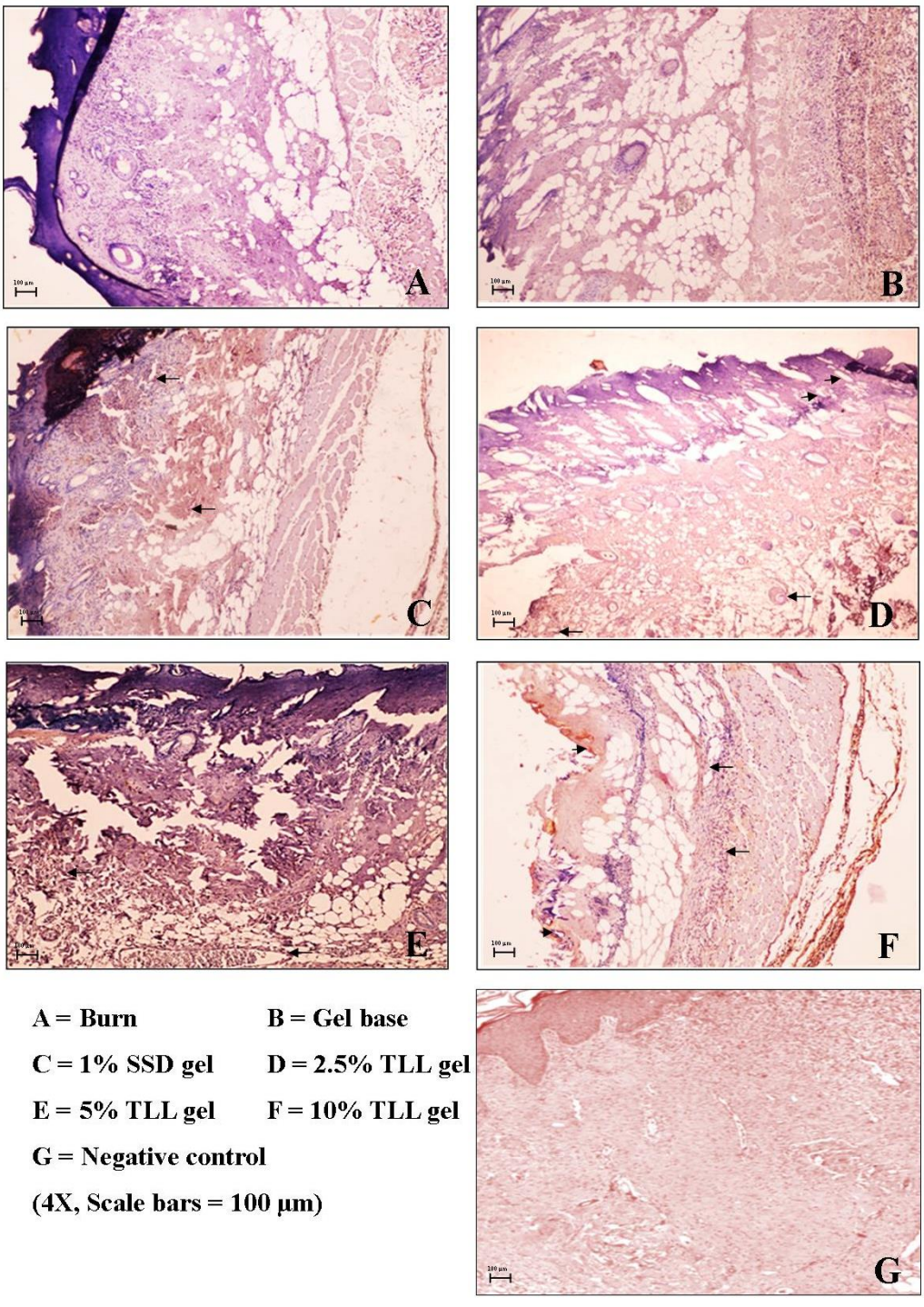


Figure 29. Immunohistochemistry analysis of skin wound. The sections were stained with monoclonal antibody for VEGF on day 3 post burning. Arrows indicated VEGF staining (brown color) (n=3).

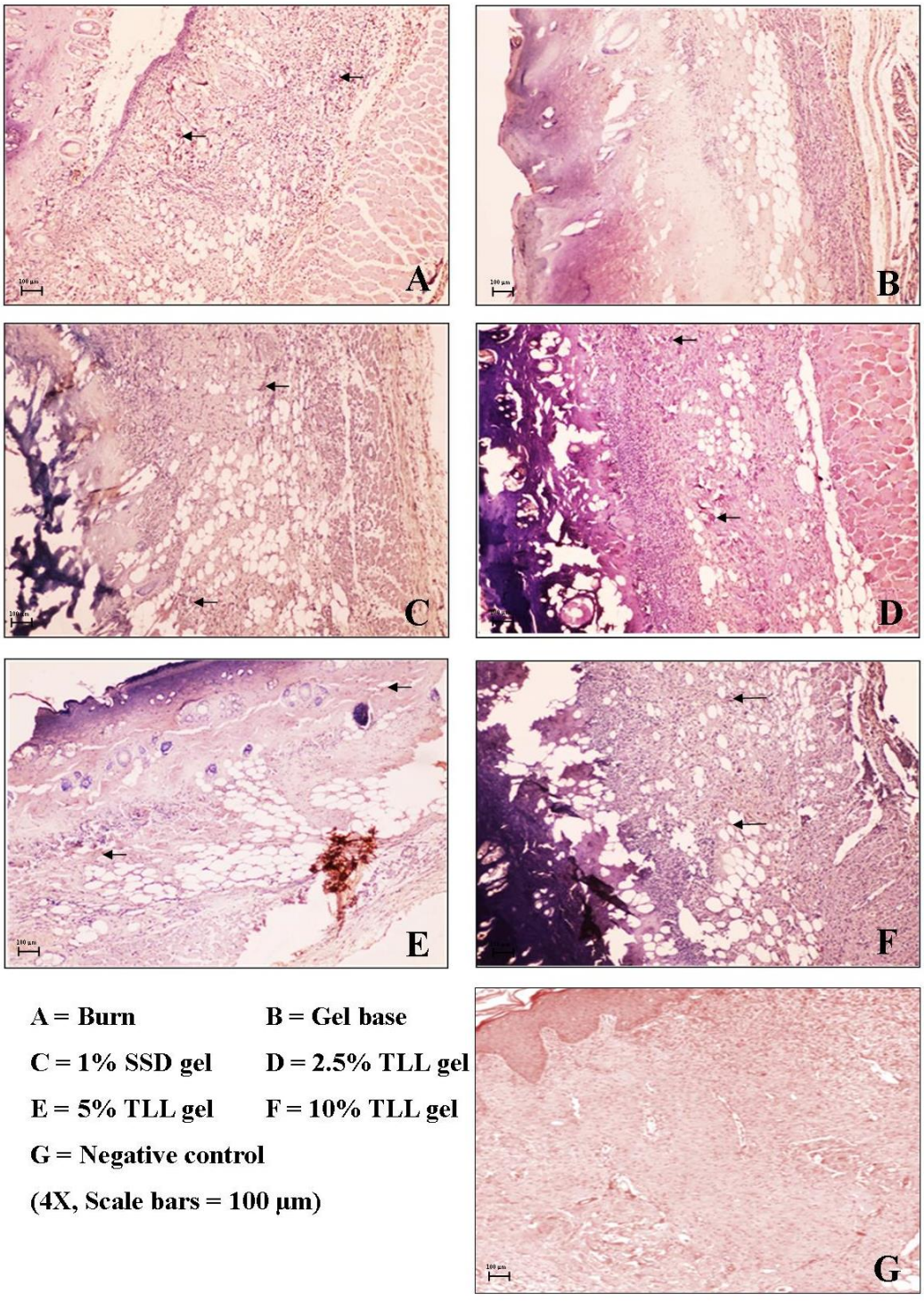


Figure 30. Immunohistochemistry analysis of skin wound. The sections were stained with monoclonal antibody for VEGF on day 7 post burning. Arrows indicated VEGF staining (brown color) (n=3).

Table 8. Immunohistochemistry scores on day 3 and day 7 in burn wound rats after topical application of TLL gel.

Experiment groups (n=3)	Day 3			Day 7		
	COX-2	TNF-α	VEGF	COX-2	TNF- α	VEGF
Burn	+	-	-	+++	++	+
Gel base	+	-	-	+	+	–
1% SSD	+	++	++	+	+	+
2.5% TLL	++	+++	+	++	++	+
5% TLL	+	++	+	+	+	+
10% TLL	++	++	+	+	++	+

+ slight; ++ moderate; +++ marked; ++++ extensive; - absent

4.6 Effects of TLL gel in second degree burn wound rats by collagen content analysis.

The wound collagen content was determined by Sircol Collagen Assay Kit, which measured as micrograms total collagen (type I-V) per milligrams tissue wet weight.

On day 3 post burning, the collagen content in TLL treated groups seemed to be increased but they did not show any significant difference from burn group (Table 9 and Figure 31).

However on day 7 and day 14 post burning, the collagen content in the wound tissue was marked increased in 10% TLL gel treated group to 17.85 ± 4.25 μg/mg tissues and 32.30 ± 6.40 μg/mg tissues respectively and it showed significant difference from burn group ($P<0.01$) (Table 9 and Figure 31).

Table 9. Collagen content (μg/mg tissues) measured on days 3, 7, and 14 of the second degree burn wound rat treated with TLL gel.

Groups	collagen content (μg/mg tissues)		
	Day 3	Day 7	Day 14
Burn	9.42±2.09	12.10±2.72	14.43±7.00
Gel base	10.10±2.45	10.82±2.97	16.17±7.04
1% SSD	13.57±3.81	14.32±4.48	17.13±5.58
2.5% TLL	11.30±5.71	14.68±1.74	20.48±5.43
5% TLL	14.17±5.88	14.55±4.08	17.87±4.69
10% TLL	16.43±6.07	17.85*±4.25	32.30*±6.40

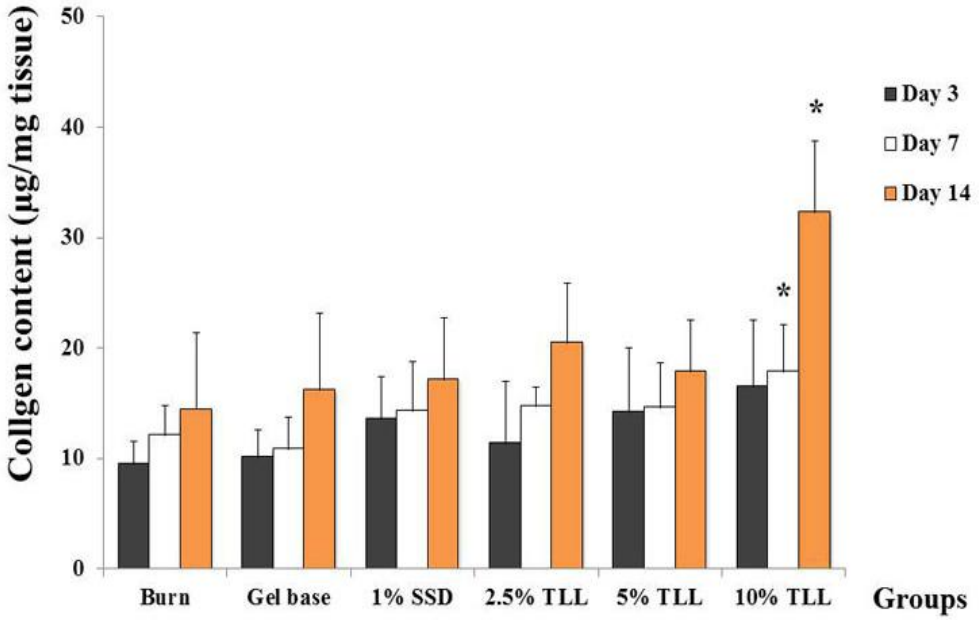


Figure 31. Effects of TLL gel on collagen content on day 3, day 7, and day 14 post treatment in second degree burn wound rats. Data are presented as mean ± SD. *P < 0.01compared to untreated group (n=6).

CHAPTER V
DISCUSSION AND CONCLUSION

Burn wound healing is a tissue repair process involving three overlapping phases of inflammatory, proliferative, and remodeling. In a large wound, thermal injury causes trauma, promotes a hypermetabolic response, and also creates ports of entry for local and systemic pathogens, resulting in microbial infection (88). It has been accepted that acceleration of wound repair is the best treatment for all varieties of wounds, as well as burn wounds. Some herbal plants have been conventionally used in burn wound treatment according to their antimicrobial, anti-oxidation or anti-inflammatory activities. These include *Aloe vera, Centella asiatica* (89, 90) etc. However, an attempt to determine more effective substances with low undesirable effects for burn wound treatment has never been declined.

In this study, rats with deep superficial second degree wound burns were initiated with a 90 degree celsius metal rod (2 cm diameters) which covered approximately 2.5 % of the total body surface area of the animals (91). The general appearances of burn wound tissues were erythema, blister, moist and blanch with pressure. Normally, the second degree wound limits itself for healing in three or more weeks. The accelerating activity of TLL extract in 10% TLL gel was clearly demonstrated in this study. TLL gel significantly increased the percentage of wound closure as early as day 4 post burning when compared to untreated burn group. The epithelialization period was also shortened to 23.83 ± 0.65 days in 10% TLL gel group compared to 30.17 ± 0.48 days in the untreated burn group. An increase in epithelialization rate in the 10% TLL group was also confirmed by histological study, collagen content in wound lesion and immunohistochemistry study.

The inflammatory phase of burn wounds is initiated by neutrophils and followed by macrophages to remove microorganisms and cell debris at the tissue injury site. These cells not only eliminate pathogens and dead cells, but also produce several inflammatory mediators, such as pro-inflammatory cytokines (TNF-α, IL-1, and IL-6), nitric oxide (NO), and prostaglandin E_2 (PGE_2). These inflammatory mediators cause pain, fever, vasodilation and an increase in vascular permeability (92). It has been reported that *T. laurifolia* leaf extract inhibited production of several inflammatory mediators in LPS-activated macrophage RAW 264.1 cells (75). *T. laurifolia* leaf extract also demonstrated anti-inflammatory activity by reducing carageenan-induced paw edema in mice (73). There was no edema or congestion on day 3, post-operation in all TLL gel treated groups but these signs of inflammation were observed in the untreated burn group. Congestion was observed until day 7 in the untreated group. A reduction in the signs of inflammation may indicate anti-inflammatory effects which may be a factor in the wound acceleration properties of TLL gel. Infiltrated inflammatory cells in wound tissues observed on day 3 and day 7 were reduced more quickly in all TLL gel treated groups (Table 7, Figure 19, 20) as compared to untreated burn group. TLL gel may shorten the inflammatory phase of the wound healing process.

The proliferative phase is characterized by angiogenesis, collagen deposition, granulation tissue formation, epithelialization, and wound contraction (14, 93). In this phase, fibroblasts and their matrix proteins, especially collagens, are essential for tissue repair and remodeling. Fibroblasts migrate into the wound area and extensively proliferate. They produce the matrix proteins, hyaluronan, fibronectin, proteoglycan, and collagen type I and III. These proteins are important for wound repair (38). Concurrently, re-epithelialization of the epidermis occurs, in which epithelial cells proliferate covering the new tissue and lead to wound closure (74). Therefore

shortened period of re-epithelialization helps accelerate wound healing. Furthermore, the process of angiogenesis occurs in conjunction with fibroblast proliferation, extracellular matrix synthesis and epithelialization in order to supply oxygen and nutrients. In this study, migrated fibroblasts were found in all burn groups on day 3, post-operation. The increase in these cells on day 7 and day 14 was higher in the TLL gel treated groups, especially in the 10% TLL gel treated group as compared to untreated group. The fibroblast proliferation was correlated to collagen content in wound tissue. Collagen content in wound tissue of 10% TLL gel treated group was higher than untreated group. TLL gel may play a role in fibroblast proliferation. In addition, angiogenesis together with epithelialization were pronounced increased in all TLL treated groups on day 14 post treatment. Increases in both fibroblasts and collagen content in wound tissue correlated well with the shortened period of epithelialization of wound skin and marked angiogenesis in the 10% TLL gel treated group.

It has been reported that stimulation of some growth factors like transforming growth factors (TGF), vascular endothelial growth factors (VEGF) leading to fibroblast proliferation and secretion of extracellular matrix (ECM) especially collagen type 1 and type III and they are partially differentiate into myofibroblast to accelerate wound healing. Tomasek et al. (2002) reported that VEGF released from the wound epithelium and the extracellular matrix by endothelial-derived proteases, stimulates endothelial cell proliferation and increases vascular permeability (54, 94).

The immunohistochemistry results from this study showed that all TTL treated groups along with silver sulfadiazine treated groups elicited a significant effect on VEGF expression on day 3 and lasted long till day 7 post treatment. Notably, VEGF expression in untreated burn group was found later in the tissue section on day 7 post wounding which conformed to histopathological finding. These results indicated that TLL gel

possessed wound healing effect on both histopathological changes and cytokine response after burn. It appeared that COX-2 expression, an inducible enzyme, responded to production of key inflammatory cytokines like prostaglandin E_2, was increased in control burn group on day 3 and prominently increased on day 7 post burning. While COX-2 expression in TLL treated groups were found almost at the same level and were in a lesser amount compared to control burn group from day 3 to day 7 post burning. Decreased COX-2 expression can down regulate inflammation and there by facilitating wound healing property of TLL. However there are some controversy results for the role of COX-2 in wound healing. For examples two wound healing studies showed that COX-2 inhibitors affect cutaneous repair (95, 96) and one report showed no significant effect of either COX-1 or COX-2 inhibition (Muller-Decker et al., 2002). The true mechanism involved in these findings should be further explored (97).

Apart from playing part as pro-inflammatory cytokine during inflammatory phase of burn, TNF-α also involves in the proliferative phase which occurs overlapping between inflammatory and proliferative phase. There have been contradictory evidences of its role in collagen synthesis and angiogenesis. Solis-Herruzo JA (1988) found that TNF-α reduced transcription of collagen α I(I) gene *in vitro* (98). Buck M (1991) showed the *in vivo* model of nude mice implanted with TNF-α producing cells exerted a reduction in collagen synthesis and collagen αI (I) gene mRNA in the skin and delayed wound healing of skin (99). In contrary, Frank et al. (2003) demonstrated that TNF-α accelerated wound epithelialization and neovascularization in full thickness dermal wound in mice (54). In addition, the differing results were also obtained in angiogenesis of TNF-α. Frater-Schroder et al (1987) found that TNF-α was a potent inhibitor of endothelial cell growth *in vitro* (56). The reverse results were found by Jakuboski et al. (2002) that TWEAK, a member of tumor necrosis factors

family, comprised pro-angiogenic activity via inducing IL-8 production in epithelial tumor cell line, increased proliferation of human vascular cell types and neovascularization in rat corneas (100). The discrepancies of these findings were based on numerous factors eg. cytokines expression in a specific organ, the exposure time and the study models etc. The immunohistochemistry results from this present study demonstrated that TNF-α level in all TLL treated groups were present from day 3 till day 7 post burning paralleled to the increase level of VEGF at the corresponding days. Even the conflicting roles of TNF-α in angiogenesis does not resolved, an increase production in proliferative phase might support its role on facilitating angiogenesis and thus improve wound healing.

Conclusion

This present study indicates that the gel from SC-CO_2 extract of *T. laurifolia* possessed wound healing property via several actions. They included improving sign of inflammation in histopathological finding, shortening time for epithelialization, increasing fibroblasts and collagen content along with angiogenesis and increased expression of VEGF from wound lesion in proliferative phase especially in 10% TLL gel treated rats. Further researches are required to validate its therapeutic efficacy as well as its safety to provide a complete preclinical wound healing model before progression to a clinical study.

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APPENDIX A

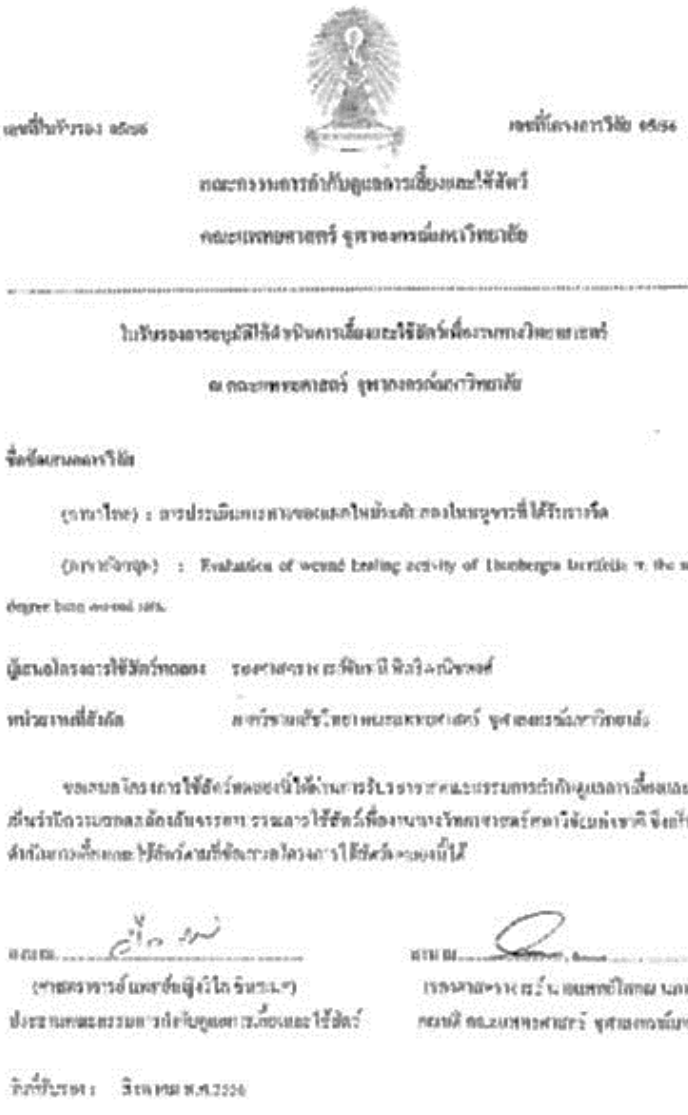

Figure 32. Certificate of animal ethic approval

APPENDIX B

.

Table 10. Percentage of wound closure measured in burn group on day 1 to day 28 post burning

Day / Burn	1	4	7	10	13	16	19	22	25	28
N1	-6.120	11.980	15.704	23.402	30.605	49.977	57.197	65.376	70.280	84.231
N2	-13.731	12.377	17.025	22.642	34.629	55.182	66.574	70.818	88.460	96.728
N3	-10.767	-5.559	6.987	15.440	27.957	62.027	75.198	83.535	87.046	95.097
N4	-27.246	-6.486	13.521	27.229	33.081	53.754	60.378	77.178	92.440	98.028
N5	-12.418	3.654	19.552	30.559	38.971	50.704	58.108	70.671	90.026	93.082
N6	-18.211	-4.900	5.886	26.699	34.965	44.662	59.947	70.906	89.307	94.495
Mean	-14.749	1.844	13.113	24.329	33.368	52.718	62.900	73.081	86.260	93.610
SD	7.279	8.794	5.539	5.207	3.809	5.836	6.860	6.342	8.031	4.909

.

Table 11. Percentage of wound closure measured in gel base group on day 1 to day 28 post burning

Day / Gel base	1	4	7	10	13	16	19	22	25	28
N1	-14.551	12.153	13.803	22.423	39.324	58.758	66.211	68.194	76.333	88.150
N2	-6.120	15.772	15.704	23.402	30.605	49.977	57.197	65.376	84.296	95.909
N3	-13.731	12.377	17.025	22.642	34.629	55.182	66.574	70.818	88.460	96.728
N4	-31.378	-7.481	10.145	22.938	35.001	56.553	63.135	89.613	100.000	100.000
N5	-11.524	3.270	14.665	24.748	37.319	49.640	64.737	81.191	91.936	96.766
N6	-17.123	-0.448	18.860	24.047	36.795	48.726	65.771	77.987	91.432	98.236
Mean	-15.738	5.940	15.034	23.367	35.612	53.139	63.937	75.530	88.743	95.965
SD	8.511	8.997	2.987	0.891	2.983	4.222	3.527	9.117	7.975	4.092

Table 12. Percentage of wound closure measured in 1% SSD group on day 1 to day 28 post burning

Day / 1%SSD	1	4	7	10	13	16	19	22	25	28
N1	-4.519	21.266	10.286	23.571	37.775	54.183	62.600	75.986	88.020	97.207
N2	-9.513	10.524	12.287	26.855	42.675	56.330	72.576	79.312	89.509	100.000
N3	-6.489	9.573	20.318	30.377	37.505	52.737	58.193	83.504	90.602	97.518
N4	-18.561	-6.382	9.046	23.764	47.265	71.898	87.851	97.708	100.000	100.000
N5	-7.712	10.707	21.244	34.059	39.571	53.364	63.888	78.457	88.594	92.652
N6	-2.603	11.615	17.027	31.894	44.223	56.215	69.986	92.349	100.000	100.000
Mean	-8.233	9.550	15.035	28.420	41.502	57.455	69.182	84.553	92.787	97.896
SD	5.605	8.918	5.223	4.367	3.888	7.227	10.515	8.637	5.655	2.877

.

Table 13. Percentage of wound closure measured in 2.5 % TLL group on day 1 to day 28 post burning

Day / 2.5%TLL	1	4	7	10	13	16	19	22	25	28
N1	-15.386	13.717	25.080	39.720	41.333	63.403	79.702	93.863	96.681	100
N2	-13.653	8.909	17.851	29.752	45.602	62.155	70.764	89.991	96.444	100
N3	-10.259	6.345	13.736	30.851	55.604	59.103	62.668	78.368	90.748	100
N4	-30.689	-0.078	11.694	21.748	33.197	46.789	64.233	78.006	96.867	100
N5	-14.569	5.143	26.879	33.307	39.124	57.664	66.814	86.339	94.977	100
N6	-22.155	2.991	16.929	30.562	43.724	55.443	58.465	74.622	95.102	100
Mean	-17.785	6.171	18.695	30.990	43.097	57.426	67.107	83.532	95.137	100
SD	7.422	4.789	6.086	5.809	7.483	5.967	7.413	7.654	2.297	0

Table 14. Percentage of wound closure measured in 5 % TLL group on day 1 to day 28 post burning

Day / 5%TLL	1	4	7	10	13	16	19	22	25	28
N1	-14.746	13.290	21.369	31.319	39.095	47.978	68.291	94.257	100.000	100
N2	-5.698	18.310	22.341	30.965	43.318	56.636	69.691	79.837	96.818	100
N3	-4.011	15.372	24.317	32.869	50.216	58.939	84.782	87.869	94.869	100
N4	-11.931	14.594	24.663	31.138	39.021	65.196	75.704	92.945	100.000	100
N5	-8.739	16.084	21.105	27.284	37.391	58.673	73.000	87.444	96.895	100
N6	-12.295	14.128	18.898	28.005	45.477	55.410	62.329	81.616	97.388	100
Mean	-9.570	15.296	22.116	30.263	42.420	57.139	72.299	87.328	97.662	100
SD	4.156	1.766	2.159	2.151	4.875	5.615	7.618	5.807	2.005	0

Table 15. Percentage of wound closure measured in 10 % TLL group on day 1 to day 28 post burning

Day / 10%TLL	1	4	7	10	13	16	19	22	25	28
N1	-14.807	15.594	23.567	39.081	57.139	65.596	71.037	94.301	100	100
N2	-5.674	20.995	24.887	39.891	51.197	70.971	88.318	96.892	100	100
N3	-5.654	19.491	33.138	42.028	56.200	84.257	96.024	100.000	100	100
N4	-12.606	11.167	22.493	28.488	40.518	60.885	71.166	92.480	100	100
N5	-13.750	15.361	25.947	33.183	52.288	64.881	70.154	93.301	100	100
N6	-2.603	13.747	17.027	31.894	44.223	56.215	69.986	93.350	100	100
Mean	-9.182	16.059	24.510	35.761	50.261	67.134	77.781	95.054	100	100
SD	5.143	3.637	5.246	5.326	6.617	9.733	11.419	2.864	0	0

Table 16. Standard collagen content in varying concentration measured at 540 nm.

Std. collagen (µg/ml)	OD 540	OD 540	OD 540	Mean ± SD
0	0	0	0	0
10	0.098	0.113	0.106	0.106 ± 0.08
20	0.186	0.225	0.206	0.206 ± 0.020
30	0.283	0.25	0.267	0.267± 0.017
40	0.267	0.3	0.284	0.283 ± 0.017
50	0.527	0.495	0.511	0.511 ± 0.016

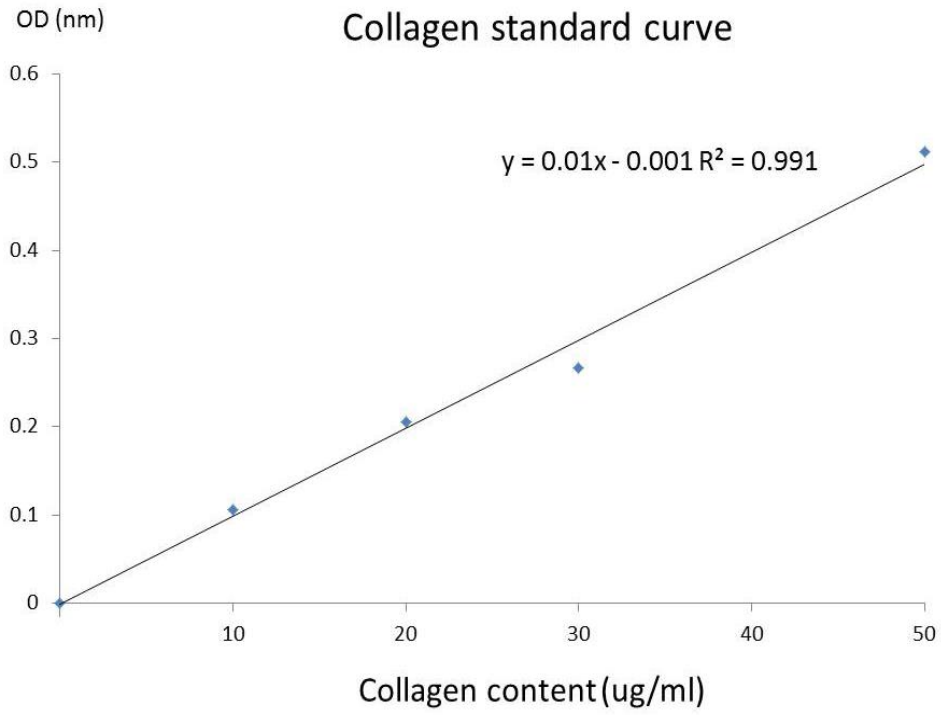

Figure 33. Standard curve of collagen concentration (µg/ml) measured at 540 nm.

Table 17. Collagen content of tissue burn wound on day 3 post burning (n=6).

N	Normal OD	Collagen (µg/mg)	Blank OD	Burn OD	Collagen (µg/mg)	Gel base OD	Collagen (µg/mg)	1% SSD OD	Collagen (µg/mg)	2.5% TLL OD	Collagen (µg/mg)	5% TLL OD	Collagen (µg/mg)	10%TLL OD	Collagen (µg/mg)
1	0.147	14.800	0.045	0.072	7.300	0.146	14.700	0.099	10.000	0.072	7.300	0.116	11.700	0.194	19.500
2	0.147	14.800	0.043	0.11	11.100	0.103	10.400	0.153	15.400	0.057	5.800	0.073	7.400	0.103	10.400
3	0.111	11.200	0.027	0.072	7.300	0.076	7.700	0.116	11.700	0.081	8.200	0.085	8.600	0.082	8.300
4	0.171	17.200	0.036	0.084	8.500	0.086	8.700	0.09	9.100	0.103	10.400	0.223	22.400	0.236	23.700
5	0.165	16.600	0.036	0.098	9.900	0.099	10.000	0.171	17.200	0.207	20.800	0.168	16.900	0.158	15.900
6	0.145	14.600	0.035	0.123	12.400	0.09	9.100	0.179	18.000	0.152	15.300	0.179	18.000	0.207	20.800
mean	0.148	14.867	0.037	0.093	9.417	0.100	10.100	0.135	13.567	0.112	11.300	0.141	14.167	0.163	16.433
SD	0.021	2.096	0.006	0.021	2.067	0.024	2.449	0.038	3.805	0.057	5.708	0.059	5.879	0.061	6.068

Table 18. Collagen content of tissue burn wound on day 7 post burning (n=6).

N	Normal OD	Collagen (µg/mg)	Blank OD	Burn OD	Collagen (µg/mg)	Gel base OD	Collagen (µg/mg)	1% SSD OD	Collagen (µg/mg)	2.5% TLL OD	Collagen (µg/mg)	5% TLL OD	Collagen (µg/mg)	10%TLL OD	Collagen (µg/mg)
1	0.147	14.800	0.045	0.123	12.400	0.08	8.100	0.196	19.700	0.174	17.500	0.209	21.000	0.244	24.500
2	0.147	14.800	0.043	0.138	13.900	0.084	8.500	0.177	17.800	0.155	15.600	0.161	16.200	0.138	13.900
3	0.111	11.200	0.027	0.105	10.600	0.154	15.500	0.159	16.000	0.143	14.400	0.107	10.800	0.204	20.500
4	0.171	17.200	0.036	0.161	16.200	0.112	11.300	0.143	14.400	0.132	13.300	0.155	15.600	0.141	14.200
5	0.165	16.600	0.036	0.083	8.400	0.085	8.600	0.088	8.900	0.125	12.600	0.139	14.000	0.148	14.900
6	0.145	14.600	0.035	0.11	11.100	0.128	12.900	0.09	9.100	0.146	14.700	0.096	9.700	0.19	19.100
mean	0.148	14.867	0.037	0.120	12.100	0.107	10.817	0.142	14.317	0.146	14.683	0.145	14.550	0.178	17.850
SD	0.021	2.096	0.006	0.027	2.723	0.030	2.972	0.045	4.483	0.017	1.738	0.041	4.082	0.043	4.253

Table 19. Collagen content of tissue burn wound on day 14 post burning (n=6).

N	Normal OD	Collagen (µg/mg)	Blank OD	Burn OD	Collagen (µg/mg)	Gel base OD	Collagen (µg/mg)	1% SSD OD	Collagen (µg/mg)	2.5% TLL OD	Collagen (µg/mg)	5% TLL OD	Collagen (µg/mg)	10%TLL OD	Collagen (µg/mg)
1	0.147	14.800	0.045	0.11	11.100	0.079	8.000	0.145	14.600	0.102	10.300	0.106	10.700	0.294	29.500
2	0.147	14.800	0.043	0.131	13.200	0.089	9.000	0.13	13.100	0.207	20.800	0.177	17.800	0.278	27.900
3	0.111	11.200	0.027	0.063	6.400	0.173	17.400	0.108	10.900	0.204	20.500	0.183	18.400	0.372	37.300
4	0.171	17.200	0.036	0.27	27.100	0.19	19.100	0.263	26.400	0.263	26.400	0.245	24.600	0.376	37.700
5	0.165	16.600	0.036	0.162	16.300	0.27	27.100	0.199	20.000	0.231	23.200	0.203	20.400	0.383	38.400
6	0.145	14.600	0.035	0.124	12.500	0.163	16.400	0.177	17.800	0.216	21.700	0.152	15.300	0.229	23.000
mean	0.148	14.867	0.037	0.143	14.433	0.161	16.167	0.170	17.133	0.204	20.483	0.178	17.867	0.322	32.300
SD	0.021	2.096	0.006	0.070	6.998	0.070	7.040	0.056	5.587	0.054	5.434	0.047	4.690	0.064	6.404

Reagents for laboratory experiments

10% neutral buffered formalin, pH7

- 37% formaldehyde 100 ml
- Na_2HPO_4 6.5 g
- $Na_2PO_42H_2O$ 4.2 g
- DW 900 ml

  Adjust pH to 7.0 and bring to 1,000 ml

PBS pH 7

- NaCl 32 g
- KCL 0.8 g
- $KH_2HPO_42H_2O$ 7.12 g
- DW 1,000 ml

  Adjust pH to 7 by HCL

10% Skim milk

- Skim milk 0.1 g
- PHS pH7 10 ml

Biebrich Scarlet-acid fuchsin solution

- Biebrich scarlet, aqueous 1% 90 ml
- Acid fuchsin, aqueous 1% 10 ml
- Glacial acetic acid 1ml

Phosphomolybdic-phosphotungstic acid solution

- Phosphomolybdic acid 5 g
- phosphotungstic acid 5 g
- DW 200 ml
-

Aniline blue solution

- Aniline blue 2.5 g
- Glacial acetic acid 2 ml
- DW 100
-

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