ผลของสารสกัดมาตรฐานบัวบก อีซีเอ 233 ต่อการสมานแผลที่เกิดจากการกรีดในหนูแรทปกติและหนูแรทที่ถูกเหนี่ยวนำให้เป็นเบาหวาน

นางสาว หทัยชนก ทันอินทรอาจ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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EFFECTS OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 ON INCISION WOUND HEALING IN NON-DIABETIC AND DIABETIC RATS

Miss Hataichanok Tanintaraard

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Thesis Title	EFFECTS OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa233 ON INCISION WOUND HEALING IN NON-DIABETIC AND DIABETIC RATS
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หทัยชนก ทันอินทรอาจ : ผลของสารสกัดมาตรฐานบัวบก อีซีเอ 233 ต่อการสมานแผลที่เกิดจากการกรีดในหนูแรทปกติและหนูแรทที่ถูกเหนี่ยวนำให้เป็นเบาหวาน. (EFFECT OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 ON INCISION WOUND HEALING IN NON-DIABETIC AND DIABETIC RATS) อ.ที่ปรึกษาวิทยานิพนธ์หลัก : รศ.ดร.มยุรี ตันติสิระ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม : รศ.ดร.บุญยงค์ ตันติสิระ 89 หน้า.

งานวิจัยนี้เป็นการศึกษาผลของสารสกัดมาตรฐานบัวบก อีซีเอ 233 ต่อการสมานแผลที่เกิดจากการกรีดในหนูแรทปกติและหนูแรทที่ถูกเหนี่ยวนำให้เป็นเบาหวาน โดยการวัดค่าแรงดึงสูงสุดที่ทำให้แผล แยกจากกัน วัดปริมาณคอลลาเจนบริเวณแผล วัดความหนาของชั้นหนังกำพร้าและศึกษาการเปลี่ยนแปลงทาง จุลพยาธิวิทยา ในวันที่ 3 และในวันที่ 7 หลังจากเป็นแผลกรีด

จากผลการทดลองพบว่าในวันที่ 3 หนูในกลุ่มปกติที่ได้รับสารสกัดมาตรฐานบัวบก 0.05% สามารถเพิ่มค่าแรงดึงสูงสุด ที่ทำให้แผลแยกจากกันได้อย่างมีนัยสำคัญทางสถิติ (5.33 ± 1.78 N/cm^2) เมื่อเทียบกับกลุ่มที่ไม่ได้รับสารทดสอบใดๆ (2.40 ± 0.41 N/cm^2) และกลุ่มที่ได้รับเจลที่ไม่มีสารทดสอบ (2.31 ± 0.39 N/cm^2) และพบว่ากลุ่มที่ได้รั บเจลสารสกัดมาตรฐานบัวบก 0.05%, 0.1% และ 0.5% สามารถเพิ่มค่าความหนาของชั้นหนังกำพร้ าได้อย่างมีนัยสำคัญทางสถิติ อีกด้วย (158.13 ± 11.38 μm, 117.71 ± 6.33 μm, 114.58 ± 8.25 μm ตามลำดับ) เมื่อเทียบกับกลุ่มที่ไม่ได้รับสารทดสอบใดๆ (81.13 ± 7.23 μm) และกลุ่มที่ได้รับเจลที่ไม่มีสารทดสอบ (88.25 ± 9.66 μm) จากการสังเกตการเปลี่ยนแปลงทางจุลพยาธิวิทยา ยังพบว่ากลุ่มที่ได้รับสารสกัดมาตรฐานบัวบก 0.05% มีแนวโน้มที่จะหายดีกว่ากลุ่มอื่น โดยพบการกระจายของไฟโบรบลาส และมีการสร้างเนื้อเยื่อใหม่ กระจายตัวอย่างหนาแน่นอยู่ในบาดแผล รวมทั้งมีการสร้างชั้นหนังกำพร้าขึ้นมาปิดคลุมแผล แต่อย่างไรก็ตาม ไม่พบการเปลี่ยนแปลงของปริมาณคอลลาเจนในกลุ่มต่างๆอย่างมีนัยสำคัญทางสถิติ ส่วนผลการทดลองในวันที่ 7 พบว่าหนูในกลุ่มต่างๆไม่มีการเปลี่ยนแปลงของค่าแรงดึงสูงสุดที่ทำให้แผลแยกจากกันและค่าความหนาของชั้นหนังกำพร้า รวมถึง ปริมาณคอลลาเจน อย่างมีนัยสำคัญทางสถิติ นอกจากนี้ยังพบประสิทธิผลในการสมานแผลกรีดของเจลสารสกัดมาตรฐานบัวบก ในหนู กลุ่มที่ถูกเหนี่ยวนำให้เป็นเบาหวา น แต่อย่างไรก็ตามการสมานแผลจะเกิดขึ้นได้ช้ากว่าในกลุ่มปกติ โดยพบว่าในวันที่ 3 หนูที่ได้รับ การทา แผลด้วยสารสกัดมาตรฐานบัวบก 0.05% ไม่พบการเปลี่ยนแปลงอย่างมีนัยสำคัญทางสถิติของ ค่าแรงดึงสูงสุดที่ทำให้แผลแยกจากกัน และ ค่าความหนาของชั้นหนังกำพร้า แต่ในวันที่ 7 พบว่าหลังการทาแผลด้วยสารสกัดมาตรฐานบัวบก 0.05% จะมีค่าแรงดึงสูงสุดที่ทำให้แผลแยกจากกัน (12.77 ± 1.45 N/cm^2) เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ เมื่อเปรียบเทียบกับกลุ่มที่ไม่ได้รับการรักษา (7.55 ± 0.36 N/cm^2) และกลุ่มที่ได้รับเจลที่ไม่มีสารทดสอบ (6.98 ± 0.95 N/cm^2) นอกจากนี้ จากการสังเกตการเปลี่ยนแปลงทางจุลพยาธิวิทยา ยังพบว่าหนูกลุ่มที่ได้รับสารสกัดมาตรฐานบัวบก 0.05% มีค่าความหนาของชั้นหนังกำพร้าเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ (137.54 ± 1.92 μm) และยังพบการกระจายของไฟโบรบลาสมากและมีการสร้างเนื้อเยื่อใหม่อีกด้วย แต่อย่างไรก็ตาม ไม่พบการเปลี่ยนแปลงของปริมาณคอลลาเจนในกลุ่มต่างๆอย่างมีนัยสำคัญทางสถิติแต่อย่างใด

ในภาวะที่เป็นเบาหวานจะมีระดับออกซิเดทีพสเตทสูงขึ้น ซึ่งอาจจะส่งผลให้การสมานแผลเกิดขึ้นได้ช้า แต่อย่างไรก็ตาม การทาแผลด้วยสารสกัดมาตรฐานบัวบก ในขนาด 0.05% สามารถเร่งการสมานแผลกรีดได้ ทั้งในภาวะปกติและในภาวะที่เป็นเบาหวาน โดยพบการเพิ่มค่าแรงดึงสูงสุดที่ทำให้แผลแยกจากกัน โดยไม่มีผลต่อการสังเคราะห์ คอลลาเจน และยัง กระตุ้นการสร้างผิวหนังใหม่ โดยเพิ่มค่าความหนาของชั้นหนังกำพร้า ซึ่งในอนาคตอาจจะต้องมีการศึกษาถึงกลไกที่แน่ชัดของสารสกัดมาตรฐานบัวบกต่อการสมานแผลอีกต่อไป

สาขาวิชาเภสัชวิทยา ..... ลายมือชื่อนิสิต .....

ปีการศึกษา 2552 ..... ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก .....

ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์ร่วม .....

#5087222820: MAJOR PHARMACOLOGY

KEYWORDS : *CENTELLA ASIATICA* / WOUND HEALING / INCISION WOUND / DIABETIC

HATAICHANOK TANINTARAARD: EFFECT OF STANDARDIZED EXTRACT OF *CENTELLA ASIATICA* ECa 233 ON INCISION WOUND HEALING IN NON-DIABETIC AND DIABETIC RATS. THESIS ADVISOR: ASSOC.PROF. MAYUREE TANTISIRA, Ph.D., THESIS CO-ADVISOR ASSOC.PROF. BOONYONG TANTISIRA, Ph.D., 89 pp.

The present study aimed to evaluate the effect of topically applied ECa 233, a standardized extract of *Centella asiatica*, on incision wound healing in non-diabetic and diabetic rats. Determination of tensile strength, total collagen content, epidermal thickness and histological observation were made on day 3 and 7 post wounding.

On day 3 post wounding, tensile strength in non-diabetic rats treated with 0.05% ECa 233 (5.33 ± 1.78 N/cm^2) was significantly increased in comparison to those untreated (2.40 ± 0.41 N/cm^2) and gel base treated groups (2.31 ± 0.39 N/cm^2). Epidermal thickness of all animals treated with 0.05%, 0.1% and 0.5% of ECa 233 (158.13 ± 11.38 μm, 117.71 ± 6.33 μm, 114.58 ± 8.25 μm) was significantly higher than in those of untreated (81.13 ± 7.23 μm) and gel base treated groups (88.25 ± 9.66 μm). In addition, a distinct fibroblast proliferation as well as granulation tissue was clearly observed in 0.05% ECa 233 treated groups whereas they were sparsely observed on the others. However, no significant difference was found on collagen content of all groups. Despite significant differences observed on day 3, no significant difference among groups was demonstrated on any parameters measured at day 7. The results observed hereby clearly suggest wound healing activity of ECa 233 0.05%. Effectiveness of topically applied ECa 233 was also demonstrated on incision wound in diabetic rats, however, with a delay of onset. Wound healing in all experimental groups of diabetic rats were comparable on day 3 whereas comparatively significant increase of tensile strength and epidermal thickness in 0.05% ECa 233 treated group were seen on day 7. Tensile strength of 0.05% ECa 233 treated group was found to be 12.77 ± 1.45 N/cm^2 on day 7 whereas they were 7.55 ± 0.36 N/cm^2 and 6.98 ± 0.95 N/cm^2 in untreated and gel base treated groups, respectively. Histological evaluation demonstrated significantly increased of epidermal thickness (137.54 ± 1.92 μm), fibroblast proliferation and granulation tissue in 0.05% ECa 233 treated groups in comparison to the others whereas no significance was observed on collagen content.

Higher level of oxidative stress in diabetes may underlie a delayed onset of healing activity in diabetic rats. However, the mechanism that accounted for the healing effects in both non-diabetic and diabetic rats seem to be similar. Dissociation between collagen content and tensile strength excludes stimulating effect on collagen synthesis. 0.05% ECa 233 seems to exert its healing effects on incision wound at least, in part, by a stimulation of epithelialization seen as an increase in epidermal thickness. Some other underlying mechanisms remain to be further investigated.

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

Academic Year : 2009 ..... Advisor's Signature .....

Co-Advisor's Signature .....

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LIST OF ABBREVIATIONS

cm	centimeter
ºC	degree Celsius
dl	deciliter
g	gram
g/kg	gram per kilogram
kd	kilodalton
mg	milligram
mg/kg	milligram per kilogram
ml	milliliter
N/cm^2	newton per square centimeter
ng	nanogram
pg	picogram
STZ	streptozotocin
µg/ml	microgram per milliliter
µm	micrometer
FGF	fibroblast growth factor
EGF	epidermal growth factor

CHAPTER I

INTRODUCTION

The primary function of the skin is to serve as a protective barrier against the environment. Integrity of skin is lost as a result of injury or wound. A wound may be resulted from an incision, laceration, contusion, abrasion and a burn (Singer and Clark, 1999; Enoch and Leaper, 2005).

Healing is a natural restorative response to tissue injury. Wound healing is the interaction of a cascade involving a highly regulated series of biological events that include a set of co-ordinate interactions between cells in the dermis and the epidermis, associate with a series of cellular responses involving blood clotting, platelet activation, inflammatory cell infiltration, re-epithelialization and the formation of granulation tissue made up of fibroblasts and blood vessels. These processes involve four overlapping phases of hemostasis, inflammation, proliferation and remodeling (Chithra *et al.*, 1998; Deonarine *et al.,* 2007).

Chronic wounds continue to be a major clinical problem. One of the leading causes of impaired wound healing is diabetes mellitus. Diabetes mellitus is a condition which is known to be combined with a variety of connective tissue abnormalities. The skin is decreased of collagen content as a result of reduced biosynthesis and/or hastened degradation of newly synthesized collagen. In diabetic patients, a minor skin

wound often leads to chronic, non healing ulcer and finally result in infection, gangrene and even amputation (Li *et al*., 2008; Badillo *et al*., 2007).

The primary goals of the treatment of wounds are rapid wound closure and a functional and aesthetically satisfactory scar. Management of wound care to promote wound healing may be by standard dressing, infectious control and topical reagent treatment such as flucidic acid, which can inhibit gram positive bacterial infection. Providone-iodine is effective against viral, bacterial and fungal infection. Another agent, Regranex® Gel contains becaplermin, an extremity diabetic neuropathic ulcer that extend into the subcutaneous tissue or beyond and have an adequate blood supply. However, the topical reagent of Regranex® Gel related adverse effect such as rash and irritation at wound site has been reported.

Efforts are being made all over the world to discover agent that can promote healing and thereby reduce the cost of hospitalization and save the diabetic patient from amputation or other severe complications. In previous study, it was found that *Centella asiatica* (Linn.) Urban (CA) can promote wound healing in different models. Maquart *et al*. (1990) reported that a triterpene extract from CA stimulated collagen synthesis and fibroblast proliferation in fibroblast cultures. In addition, Shukla *et al*. (1999) found that asiaticoside isolated from CA increased hydroxyproline, tensile strength, collagen content, epithelization in punch wound model in normal and diabetic rats. Furthermore, the study of Kimura *et al.* (2008) showed that the application of asiaticoside at low doses of 10^{-8} to 10^{-12}% (w/w) increases MPC-1, VEGF and IL-1β in burn wound repair. Recently, by using activity guided isolation, the study has established a standardized extract of CA

ECa 233 defined as a white to off white titrated extract of CA containing at least 80% of triterpenoids and the ratio between madecassoside and asiaticoside is 1.5 ± 0.5 (Tantisira *et al*., 2009).

The present study was designed to determine the effect of ECa 233 on incision wound healing in non-diabetic and diabetic rats. The healing effects of ECa 233 were assessed using tensile strength test, biochemical analysis (total collagen content), epidermal thickness and histological examination.

Objective

To study the effect of ECa 233, standardized extract of *Centella asiatica*, on incision wound healing in non-diabetic and diabetic rats.

CHAPTER II

LITERATURE REVIEWS

1. *Centella asiatica* (Linn.) Urban

Synonyms: *Hydrocotyle* asiatica, Gotu Kola, Indian Pennywort, Indian Water Navelwort, Mandukaparni

Thai name: Bua Bok, Pa-na-e-khaa-doh, Phak wean, Phak nok

Habitat: Asia and Africa

Part used: whole plant

Figure 2.1 *Celtella asiatica* (Linn.) Urban

Centella asiatica (Linn.) Urban (Figure 2.1) has been widely cultivated as a vegetable or spice in China, Southeast Asia, Sir lanka, Africa and Oceanic countries. It appears in Sri lankan and Indian Ayurvedic traditional medicine since prehistoric time. The name hydrocotyle is derived from the Greek word for 'water' and 'cup', to

describe the habitat, water and the appearance of the leaves as cup-shaped (Matsuda *et al*.,2001; Williamson, 2002).

Table 2.1 Systematic classification of *Centella asiatica.*

Classification	Name
Kingdom	Plantae
Division	Spermatophyta
Class	Dicotyledoneae
Order	Araliales
Family	Apiaceae (Umbelliferae)
Subfamily	Hydrocotyle
Genus	Centella
Species	Asiatica

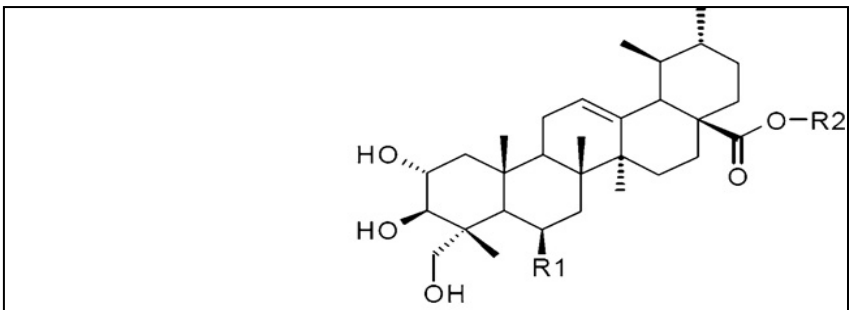
1.1 Botany

The *Centella asiatica* is a perennial, slender, herbaceous and creeper plant flowering between August and September; its flowers are of pale violet color. The plant has a smell reminiscent of tobacco leaves and a mildly bitter taste. The leaves are glabrous thin and soft, kidney shaped about 2 to 5 cm in diameter with a long petioles arising rosette-like from a common base and the individual “leaf rosettes” are connected aerial stolons or runners. The stems (stolons) are slender, prostates and often reddish colored. The 2 to 5 fruits of each umbel are closed within a pericarp comprising 1 to 2 cm large elliptical bracts (Williamson, 2002).

1.2 Chemical constituents

Centella asiatica contains a variety of organic acid, triterpenoids (asiatic acid, 6-hydroxy asiatic acid, madecassic acid, betulinic acid, thankunic acid and isothankunic acid) are present together with their glycosides up to 8% depending on countries of origin of the plant. The major saponins are asiaticoside, asiaticoside A, asiaticoside B, madecassoside, brahmoside, brahminoside, thankuniside, and isothankuniside (Williamson, 2002).

In addition, *Centella asiatica* contains essential oil about 0.1% of the aerial part and sesquiterpenoids up to 80%, with a β-caryophyllene, α-humulene and germacrene-D, elemene and bicycloelemene, trans-farnesene being the most abundant. It contains of flavone derivatives (quercetin, kaempferol glycosides and astragalin), phytosterols (strigmasterol and sitosterol). The leaf contains of aminoacids (alanine, arginine, aspartic acid, glutamic acid, glycine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, proline, threonine and tryptophan) (Kuhn and Winston, 2007).



Asiatic acid	R1 = H	R2 = H
Madecassic acid	R1 = OH	R2 = H
Asiaticoside	R1 = H	R2 = Glu-Glu-Rha
Madecassoside	R1 = OH	R2 = Glu-Glu-Rha

Figure 2.2 Structures of chemicals in *Centella asiatica*.

1.3 Traditional applications

Centella asiatica has been used for treatment of rheumatic pain, skin infections, leprosy, burns, as a diuretic, strengthening the nerves and the brain cells, to increases intelligence, longevity, memory, treat petit mal epilepsy in India's traditional ayurveda medicine. It is used for treating dermatitis, wounds, dysentery, sores, tuberculosis, jaundice, hematuria, hemoptysis, and as a nerve tonic in Chinese medicine (Kuhn and Winston, 2007).

Today, American and European herbalists used it for disorders that cause connective tissue swelling, such as scleroderma, psoriatic arthritis, anklylosing spondylitis and rheumatoid arthritis. It was first accepted as a drug in France in the 1880.

1.4 Pharmacological study

Centella asiatica exhibit various pharmacological activities including:

1.4.1 Wound healing effect

The oral and topical administration of an alcoholic extract of *Centella asiatica* on rat dermal wound healing increases DNA, protein, collagen content of granulation tissue, cellular proliferation and collagen synthesis at the wound site. It increases the rate of wound contraction, epithelialization and better maturation, collagen crosslinking (Suguna *et al.,* 1996). The ethanolic extract of *Centella asiatica* significantly increases breaking strength, rate of wound contraction and hydroxyproline content in both normal and dexamethasone suppressed wound healing model (Shetty *et al*., 2006).

The topical applications of asiaticoside, isolated from *Centella asiatica,* in different concentration 0.2%, 0.4% and oral route of asiaticoside 1mg/kg increases hydroxyproline, tensile strength, collagen content and better epithelialization on punch wounds in normal as well as delayed type wound model. It can promote angiogenesis in the chick chorioallantoic membrane model at 40 µg/disk concentration (Shukla *et al*., 1999).

Asiaticoside could promote fibroblast proliferation and extracellular matrix synthesis in wound healing. In vitro studies demonstrated that asiaticoside (30 µg/ml) significantly up-regulated gene expression, mRNA level and protein productions of certain gene responsible for extracellular matrix synthesis in a human dermal fibroblast (Lu *et al*., 2004).

Titrated extract from *Centella asiatica* (TECA) is a reconstituted mixture of 3 triterpenes that included asiatic acid, madecassic acid and asiaticoside. It was shown to increase dry weight of DNA, total protein, collagen and uronic acid contents, showing an increased remodeling of the collagen matrix and stimulate glycosaminoglycan synthesis in stainless steel wound chambers model. Asiatic acid and asiaticoside were the most active of the 3 triterpenes. Asiaticoside stimulated collagen synthesis and was active at low doses (Maquart *et al*., 1999).

The application of asiaticoside at a low dose (10 pg, 1 ng, or 100 ng/wound area) increased MPC-1expression in keratinocytes and increases IL-1β expression in macrophages level, stimulates vascular epithelial growth factor level in burn wound model (Kimura *et al.,* 2008).

1.4.2 Anti-ulcer effect

Oral administrations of *Centella* extract (0.05 g/kg, 0.25 g/kg and 0.50 g/kg) prevented ethanol-induced gastric mucosal lesion by inhibiting gastric lesion and decrease mucosal myeloperoxidase activity in rats (Cheng and Koo, 2000). In addition, oral administration of different concentrations of *Centella* extract and asiaticoside reduce the size of ulcer at day 3 and 7, decreases myeloperoxidase activity at the ulcer tissues. It could promote epithelial proliferation and angiogenesis, up-regulate of basic fibroblast growth factor (FGF) and important angiogenic factor expression in acetic acid induced gastric ulcers rats (Cheng *et al.,* 2004).

1.4.3 Anti-inflammatory effect

The anti-inflammatory activity of *Centella asiatica* was studied in rats using prostaglandin E_2 induced paw edema. Water extract of *Centella asiatica* at 4 and 10 mg/kg exhibited significantly different anti-inflammatory activity. This effect was similar to the non-steroidal anti-inflammatory drug, mefenamic acid (Somchit *et al.,* 2004).

1.4.4 Anti-norciceptive effect

Water extract of *Centella asiatica* (10, 30, 100 and 300 mg/kg i.p.) significantly reduced the number of writhing by approximately 13%, 45%, 64% and 85%, respectively. Intraperitoneal administration of *Centella asiatica* significantly prolonged the response latency in the hot-plate test in a dose dependent manner (Somchit *et al.,* 2004).

1.4.5 Antimicrobial and antifungal effects

The essential oil of *Centella asiatica* showed remarkable activity against Escherichia coli, Aspergillus niger, Rhizopus oryzae, Fusarium solini, Candida albicans and Colletotrichum musae (Minija and Thoppil, 2003).

The study of antimicrobial and antifungal activity of petroleum ether, ethanol and water extract of *Centella asiatica* was carried out by agar diffusion method. It was found that the zone of inhibition produced by petroleum ether, ethanol and water extract in dose of 62.5, 125, 250, 500 and 1000 μg/ml against some selected strains (P. vulgaris, S. aureus, E. coli, A. niger, C. albicans) were measured and compared with standard antibiotics ciprofloxacin (10 μg/ml) (Jagtap *et al.,* 2009).

1.4.6 Antioxidant effect

The effects of asiaticoside on the levels of certain antioxidants in the wound so as to examine the possible involvement of such a mechanism in the asiaticoside induced wound healing were studied. A topical application of 0.2% asiaticoside twice daily for 7 days to excision type cutaneous wounds in rats led to increased enzymatic and non-enzymatic antioxidants as follow; superoxide dismutase (35%), catalase (67%), glutathione peroxidase (49%), vitamin E (77%) and ascorbic acid (36%) in newly formed tissues. It also resulted in a several fold decreases in lipid peroxide levels (69%) as measured in terms of thiobarbituric acid reactive substance (Shukla *et al.,* 1999).

1.4.7 Cardiovascular effect

The cardioprotective effect of *Centella asiatica* on myocardial marker enzymes and antioxidant enzymes in adriamycin induced cardiomyopathy was evaluated in rats. Pre-co-treatment with oral administration 200 mg/kg body weight of Centella *asiatica* extract significantly prevented myocardial damage and restored the antioxidant enzymes (SOD, CAT, GPx and GST) to near normal levels (Gnanapragasam *et al.,* 2004).

1.4.8 Anticancer effect

The methanolic extract (CE) and acetone fraction (AF) of *Centella asiatica*. AF and CE inhibited the proliferation of the transformed cell line in a dose dependent manner for Ehrlich ascites tumor cells (EAC) and Dalton's lymphoma ascites tumor cells (DLA), respectively. Despite, no toxic effects were detected in normal human lymphocytes. In addition AF significantly suppressed the multiplication of mouse lung fibroblast (L-929) cells in long term culture. Oral administration of CE and AF retarded the development of solid and ascites tumors and increased the life span of these tumor bearing mice (Babo *et al.,* 1995).

The anti-tumor activities of crude water extract of *Centella asiatica* using human colon adenocarcinoma-derived Caco-2 cells were examined. *Centella asiatica* extract reduced the proliferation rate of Caco-2 cells significantly in a concentration and time dependent manner. The mechanism of cancer cell growth inhibition was shown to occur via cell cycle arrest. The extract induced S and G_2-M arrest in Caco-2 cells accompanied apoptosis induction. It also increased the accumulation of cyclin B1 protein in the cells, *Centella asiatica* extract inhibited cell proliferation of Caco-2 cells though modification of the cell cycle events and this cell

cycle arrest is associated, at least in part, with increased accumulation of cyclin B1 protein (Bunpo *et al.,* 2005).

1.4.9 Neurological effect

The neuroprotective efficacy of a standardized aqueous extract of *Centella asiatica* (CA) against 3-Nitropropionic acid (3-NPA) induced early oxidative stress and mitochondrial dysfunctions in striatum and other brain regions were studied. The neurotoxicant elicited marked oxidative stress in the untreated mice as evidenced by elevated levels of malondialdehyde, reactive oxygen species (ROS) and hydroperoxides in the striatum (cytosol and mitochondria) while CA prophylaxis completely attenuated the 3-NPA-induced oxidative stress. 3-NPA also caused significant oxidative stress and protein oxidation in cytosol/mitochondria of other brain regions as well which were predominantly abolished by CA prophylaxis. Significant depletion of glutathione (GSH) levels, total thiols and pertutbations in antioxidant enzymic defences in striatum and other brain regions discernible among 3-NPA administered mice were also protected with CA prophylaxis (Shinomol and Muralidhara, 2008).

Centella asiatica fresh leaf extract has neuronal dendritic growth stimulating properties. It also significantly increases the dendritic length (intersections) and dendritic branching points along the length of both apical and basal dendrites in rats treated with 6 ml/kg body weight/day of *Centella asiatica* for 6 weeks (Kappettu Gadahad *et al.,* 2007).

1.5 Pharmacokinetics

The pharmacokinetics of asiatic acid after oral administration of the total triterpenic fraction of *Centella asiatica* in single doses (30 or 60 mg) and after a 7 day treatment (30 or 60 mg twice daily) was studied. Twelve healthy volunteers received each treatment following a randomized cross-over design with trials separated by a 3 week interval. The time of peak plasma concentration was not affected by dosage difference or by treatment scheme. Differences in peak plasma concentration and area under the concentration vs. time curve from 0 to 24 h calculated after administration a single dose of the total triterpenic fraction of *Centella asiatica* 30 or 60 mg were accounted for by the different dose regimen. However, after chronic treatment with both 30 and 60 mg, peak plasma concentrations, area under the curve (AUC) 0-24 and half-life were significantly higher than those observed after the corresponding single dose administration (Grimaldi *et al.,* 1990).

1.6 Toxicology

The oral medial lethal dose (LD_{50}) of hydroalcoholic extract of *Centella asiatica* in rats was found to be higher than 675 mg/kg, indicating a high therapeutic index. In addition, chronic oral administration also exhibited a low toxicity for *Centella asiatica* (De *et al*., 1997).

Acute toxicity of standardized extract of *Centella asiatica* (ECa 233) demonstrated that oral administration of 10.0 g/kg extract into 20 mice did not cause any lethality. In addition, the extract did not cause any toxic sign. Sub-chronic toxicity study of ECa 233 has been investigated in Wistar rats. Oral administered of ECa 233 (10, 100 and 1000 mg/kg/day) for 3 months showed no difference with regards to body weight, food consumption and health in comparison to the control group. Histopathological result of internal organs did not show any incidence or

degree of lesions in a dose dependent manner with the increasing dose of ECa 233 (Tantisira *et al.,* 2008).

2. **The skin** (Marieb, 1995; Tortora, 2005)

The skin is the major organ of the integumentary system. As the body's largest organ, the skin accounts for about 15% of the body's weight. The skin also called the integument, which simply means are covering.

2.1 Structure of the skin

The two major layers of the skin are the epidermis and the dermis. The epidermis is the outer, thinner layer of skin, while the dermis is the inner, thicker layer. The epidermal-dermal junction is the region where the cells of the epidermis meet the connective tissue cells of the dermis. Beneath the dermis lies a loose subcutaneous layer rich in fat and alveolar tissue. This layer is called the superficial fascia or hypodermis.

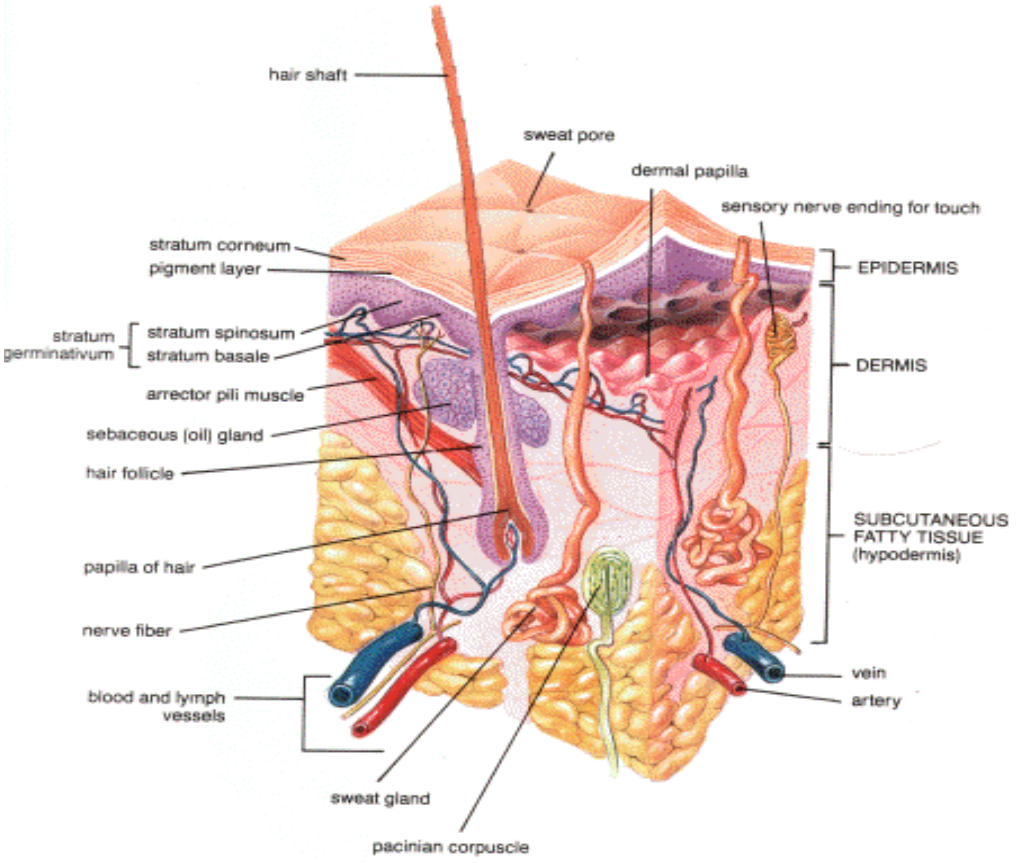


Figure 2.3 structure of the skin

2.1.1 The epidermis

The epidermis is the outermost layer of the skin. It is composed of keratinized stratified squamous epithelium consisting of four distinct cell types: keratinocytes, melanocytes, Langerhans cells and Markel cells. About 90% of epidermal cells are keratinocytes, which are arranged in four or five layers. These cells produce keratin, the fibrous protein that protects the epidermis from heat, microbes and chemicals. About 8% of epidermal cells are melanocytes, which develop from neural crest cells (ectoderm) of a developing embryo. The melanocytes produce the pigment melanin. Melanin is a brown-black pigment that

contributes to skin color and absorbs damaging ultraviolet light. The Langerhans cells arise from red bone marrow and migrate to the epidermis. They participate in immune responses mounted against microbes that invade the skin. Markel cells are the least numerous of the epidermal cells. They are located in the deepest layer of the epidermis, where they contact the flattened process of the sensory neuron, a structure called a tactile disc. Markel cell and tactile disc function in the sensation of touch.

In thick skin, which covers the palms, fingertips and soles of the feet, the epidermis consists of five layers or strata. From deep to superficial, these layers are: stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and stratum corneum. In the thin skin, which covers the rest of the body, the stratum lucidum is absent and the other four layers are thinner.

2.1.1.1 The stratum basale (Basal layer)

The deepest layer of the epidermis is firmly attached to the underlying dermis along a wavy borderline. The stratum basale is composed of a single row of cells. The many mitotic nuclei seen in this layer reflect the rapid division of these cells and account for its alternate name, stratum germinativum. Some 10% to 25% of the cells in the stratum basale are melanocytes, and their branching processes extend among the surrounding cells reaching well into the more superficial stratum spinosum layer. Occasionally Markel cells are also seen in this layer.

2.1.1.2 The stratum spinosum (Spiny layer)

Superficial to the stratum basale is the stratum spinosum, where 8-10 layers of the polyhedral keratinocytes fit closely together. The stratum spinosum is several cell layers thick. These cells contain thick bundles of intermediate filaments (tonofilaments). Tonofilaments consist of a tension-resisting protein (prekeratin). The keratinocytes in this layer are somewhat flattened and irregular in shape. When cells of the stratum spinosum are prepared for the microscopic examination, they shrink and pull apart such that they appear to be covered with thornlike spines, although they are rounded and larger in living tissue. At each spinelike projection, bundles of tonofilaments insert into desmosome, which tightly join the cells to one another. This arrangement provides both Langerhans cells and melanocytes also appear in this layer.

2.1.1.3 The stratum granulosum (granular layer)

At about the middle of the epidermis, the stratum granulosum consists of three to five layers of flattened keratinocytes. The nuclei and other organelles of these cells begin to degenerate. Along with abundant tonofilaments, these cells contain keratohyaline granules and lamellated granules. The keratohyaline granules contribute to the formation of keratin in the higher layer. The lamellated granules contain a waterproof glycolipid that is secreted into the extracellular space and is a major factor slowing water loss across the epidermis. As their nuclei break down during apoptosis, the keratinocytes of the stratum granulosum can no longer carry on vital metabolic reactions, and die. Thus, the stratum granulosum marks the transition between the deeper, metabolically active strata and the dead cells of the more superficial strata.

2.1.1.4 The stratum lucidum (Clear layer)

The stratum lucidum is present only in the thick skin of the fingertips, palms and soles. It consists of a few rows of flattened dead keratinocytes with indistinct boundaries. Here, or in the stratum corneum above, the gummy substance of the keratohyalin granules becomes intimately associated with parallel arrays of tonofilaments in the cells. This keratohyalin-tonofilament combines froms the keratin fibrils. As mentioned before, the stratum lucidum is present only in thick skin.

2.1.1.5 The stratum corneum (Horny layer)

The outermost layer, the stratum corneum, is a broad zone 20 to 30 cell layers thick. It accounts for up to three-quarters of the epidermal thickness. Keratin and the thickened plasma membranes of cells in the stratum corneum protect the skin against abrasion, penetration, injury and microbes and serves as an effective water-repellent barrier. The shingle-like cell remnants of the stratum corneum are referred to as cornified or horny cells. They are familiar to everyone as the dandruff shed from the scalp and the flakes that slough off dry skin.

2.1.2 The dermis

The dermis, the second major skin region, is a strong but flexible connective tissue layer. The cell types found in the dermis are typical of those found in any connective tissue proper: fibroblasts, macrophages,occasional mast cell, white blood cells and some adipocytes. It is a semifluid matrix heavily embedded with collagen, elastin and reticular fibers. The dermis is richly supplied with nerve

fibers, blood vessels, lymphatic vessels and hair follicles, the dermis can be divided into a superficial papillary layer and a deeper reticular layer.

2.1.2.1 The papillary layer

The papillary layer makes up about one to fifth of the thickness of the total layer. It consists of areolar connective tissue containing fine elastic fibers. Its surface area is greatly increased by small, fingerlike projections called dermal papillae that indent the overlying epidermis. These nipple-shaped structures and some contain capillary loops. Other dermal papillae contain touch receptors called corpuscles of touch or Meissner's corpuscles, which contain sensitive nerve endings. Also present in the dermal papillae are free nerve endings, dendrites lack any apparent structural specialization. Different free nerve endings initiate signals that produce sensations of warmth, coolness, pain, tickling and itching.

2.1.2.2 The reticular layer

The deeper reticular layer, which accounts for about 80% of the dermis, is typical dense irregular connective tissue. Its extracellular matrix contains thick bundles of interlacing collagen fibers and some coarse elastin fibers. The bundles of collagen fibers in the reticular layer interlace in a netlike manner. Spaces between the fibers are occupied by a few adipose cells, hair follicles, nerves, sebaceous glands and sweat glands. The collagen fibers of the dermis give skin strength and resiliency, which prevent most jabs and scrapes from penetrating the dermis. In addition, collagen binds water, helping to maintain the hydration of the skin. Elastin fibers provide the stretch-recoil properties of the skin.

2.2 Function of the skin

The skin and derivatives perform a variety of functions that affect body metabolism and prevent external factors such as bacteria, abrasion, temperature and chemicals from upsetting body homeostasis.

2.2.1 Regulation of body temperature

In response to high environmental temperature or strenuous exercise, the evaporation of sweat from the skin surface helps lower an elevated body temperature to normal. In response to low environmental temperature, production of sweat is decreased, which helps to conserve heat.

2.2.2 Protection

The skin covers the body and provides protection in various ways, constitutes at least three types of barriers: chemical, physical and biological. Keratin in the skin protects underlying tissues from microbes, abrasion, heat and chemical. Lipids released by lamellar granules retard evaporation of water from the skin surface. The oily sebum from the sebaceous glands protects skin and hairs from drying out and contain bactericidal chemical that kill surface bacteria. The pigment melanin provides some protection against the damaging effects of ultraviolet light.

2.2.3 Blood reservoir

The skin vascular supply is quite extensive and can hold large volumes of blood (about 5% of the body's entire blood volume). When other body organs,

such as vigorously working muscles, need a greater blood supply, the nervous system constricts the dermal blood vessels. This shunts more blood into the general circulation, making it available to the muscles and other body organs.

2.2.4 Cutaneous sensations

The skin is richly supplied with cutaneous sensory receptors, which are actually part of the nervous system. The cutaneous receptors are classified as exteroceptors because they respond to stimuli arising outside the body. These include tactile sensation; touch, pressure, vibration and tickling as well as thermal sensation such as warmth and coolness. Another cutaneous sensation such as pain usually is an indication of impending or actual tissue damage.

2.2.5 Metabolic function

When sunlight bombards the skin, modified cholesterol molecules in epidermal cell are converted to a vitamin D precursor. This precursor is absorbed into the dermal capillaries and transported to other body areas to play various roles in calcium metabolism.

2.2.6 Excretion and absorption

The skin plays minor roles in excretion and absorption. Besides removing water and heat, sweat is also the vehicle for excretion of small amounts of salt, carbon dioxide and two organic products of protein breakdown (ammonia and urea). The absorption of water-solution substances through the skin is negligible,

but certain lipid-soluble materials do penetrate the skin. These include fat-soluble vitamins (A, D, E and K) and oxygen and carbon dioxide gases.

3. The wound

3.1 Definition of wound

A wound may be defined as loss of the integrity of epithelium and may be accompanied by disruption of the structure and function of underlying normal tissue or tissue caused by physical, chemical or biological insults. (Enoch and Leaper, 2005)

3.2 Types of wound (Leaper and Gottrup, 1998)

3.2.1 Abrasion wound

An abrasion is a superficial injury. This type of wound damages only the epidermis and should not therefore bleed. However, abrasions do usually extend into the dermis causing slight bleeding. Abrasions are commonly caused by a glancing impact across the surface of the skin. These wounds are seen where an object has struck the skin or where the injured person has fallen onto a rough surface.

3.2.2 Contusion wound

Contusions are caused by blunt trauma or injury to tissues, resulting in damage to blood vessels beneath the surface. Blood leaks out into surrounding tissues from damaged capillaries, venules and arterioles. Contusions may be surface or deeper within tissues or organs.

3.2.3 Laceration wound

These wounds are commonly known as gashes, 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 be damaged. Lacerations therefore bleed profusely. Lacerations have ragged wound edges, as they have been torn apart and not neatly incised as in a surgical wound.

3.2.4 Incision wound

Incision wounds are sharp cut-like injuries. The edges of the wound will vary according to the nature of the cutting edge of the object. Incision wound is divided into two types, surgical incision and non-surgical incision wound. Surgical incision wounds are usually clean, except when they are made to treat an infective condition such as an abscess. Non-surgical or penetrating wounds are caused by injuries inflicted by a knife or other sharp instrument.

3.3 Wound healing

Wound healing is a natural restorative response to tissue injury. Healing is a dynamic that worked by interactive process involving soluble mediators, blood cells, extracellular matrix and parenchymal cell. Wound healing is a systematic process with four phases: hemostasis, inflammation, proliferation and remodeling (Singer and Clark, 1999; Deonarine *et al.,* 2007).

3.3.1 Hemostasis phase (immediately)

The healing cascade begins immediately following injury when the platelets come in to contact with exposed collagen. As platelet aggregation proceeds, clotting factors are released resulting in the deposition of a fibrin clot at the site of injury. The fibrin clot serves as a provisional matrix and sets the stage for the subsequent events of healing. Platelet releases the clotting factors for bleeding control as well as loss of fluid and electrolytes. It also provides a cascade of chemical signals. The two most important signals are platelet derived growth factor (PDGF) and transforming growth factor –beta (TGF-β). At the site of the platelet clot, coagulation system enzymes are activated and fibrinogen is converted to fibrin. The resulting network forms the provisional matrix for tissue repair. Several hours after clot formation, keratinocytes begin to move into the site from the edges of the injury to being to close the wound (Diegelmann and Evans, 2004; Deonarine *et al.,* 2007).

3.3.2 Inflammatory phase (day 1-5)

The second stage of wound healing is inflammation. The inflammatory cells include neutrophils, macrophages and mast cells. Neutrophils are first to infiltrate the site of the wound within 24 hours after injury. The major function of the

neutrophil is to remove foreign material, bacteria and non-functional host cells and damaged matrix components that are present in the wound site. During bacterial protein synthesis a waste product represented by a tri-peptide is released which in turn attracts inflammatory cells. Neutrophils will engorge themselves until they are filled with bacteria and constitute what is call “laudable pus” in the wound. The mast cells release granules filled with enzymes, histamine and other active amines and these mediators are responsible for the characteristic signs of inflammation around the wound site. The active amines released from the mast cell, cause surrounding vessels to become leaky and thus allow the speedy passage of the mononuclear cell into the injury area. In addition fluid accumulates at the wound site and the characteristic signs of inflammation begin. The signs of inflammation have been well recognized since ancient time: redness, heat, swelling and pain.

Tissue macrophages arrive within 48 hours after injury. These specialized wound macrophages are perhaps the most essential inflammatory cells involved in the normal healing response. Once activated these wound macrophages also produced both cytokines and growth factors. They also play a role in debridement, acting as phagocytes to clear away matrix debris. The appearance of activated macrophages is accompanied by the appearance of the lymphocytes and marks the end of the inflammatory phase and the beginning of the proliferative phase of wound healing. (Diegelmann and Evans, 2004)

3.3.3 Proliferative phase (day 3-14)

The proliferative phase is associated with the production of collagen, proteoglycans and fibronectin to from new extracellular matrix, continue epithelialization, and angiogenesis. Fibroblast, which produces matrix and collagen, is the predominant cell in this phase. The TGF- β released by the platelets, macrophages and T lymphocytes becomes a critical signal. TGF- β is considered to

be a master control signal that regulates a host of fibroblast functions. TGF- β has three effects on extracellular matrix deposition. First, it increases transcription of the genes for collagen, proteoglycans and fibronectin thus increasing the overall production of matrix proteins. At the same time TGF- β decreases the secretion of proteases responsible for the breakdown of the matrix and it also stimulates the protease inhibitor, tissue inhibitor of metallo-protease (TIMP). Other cytokines considered to be important are interleukins, fibroblast growth factors and tumor necrosis factor-alpha. As healing progresses several other important biological responses are activated. The process of epithelialization is stimulated by the presence of epidermal growth factor (EGF) and transforming growth factor alpha (TGF-α) that are produced by activated wound macrophages, platelets and keratinocytes. Once the epithelial bridge is complete, enzymes are released to dissolve the attachment at the base of the scab resulting in removal. Angiogenesis or neovascularization begin with the migration of the endothelial cells into the fibrin matrix. Endothelial cells begin to degrade the interstitial matrix in order to form new capillaries. TGF- β along with vascular endothelial growth factor (VEGF) and basic fibroblast growth factor stimulate angiogenesis (Diegelmann and Evans, 2004; Deonarine *et al.,* 2007).

3.3.4 Remodeling phase (day 7- 1 year)

The final stage of wound healing is remodeling or maturation of the scar begins during the fibroplastic phase, and is characterize by a reorganization of previously synthesized collagen. Collagen is broken down by matrix metalloproteinases (MMPs), and the net wound collagen content is the result of a balance between collagenolysis and collagen synthesis. There is a net shift toward collagen synthesis and eventually the re-establishment of extracellular matrix composed of a relatively a cellular collagen-rich scar. As the collagen matures and becomes older, more and more of these intramolecular and intermolecular cross-

links are placed in the molecules. This important cross-links step gives collagen its strength and stability over time. The deposition of matrix at the wound site follows a characteristic pattern: fibronectin and collagen type III constitute the early matrix scaffolding; glycosaminoglycans and proteoglycans represent the next significant matrix components; and collagen type I is the final matrix. By several weeks post injury the amount of collagen in the wound reaches a plateau, but the tensile strength continues to increase for several more months. The regained tensile strength in a wound will never approach normal. Intact the maximum tensile strength that a wound can ever achieve is approximately 80% of normal skin (Diegelmann and Evans, 2004; Efron *et al.,* 2007).

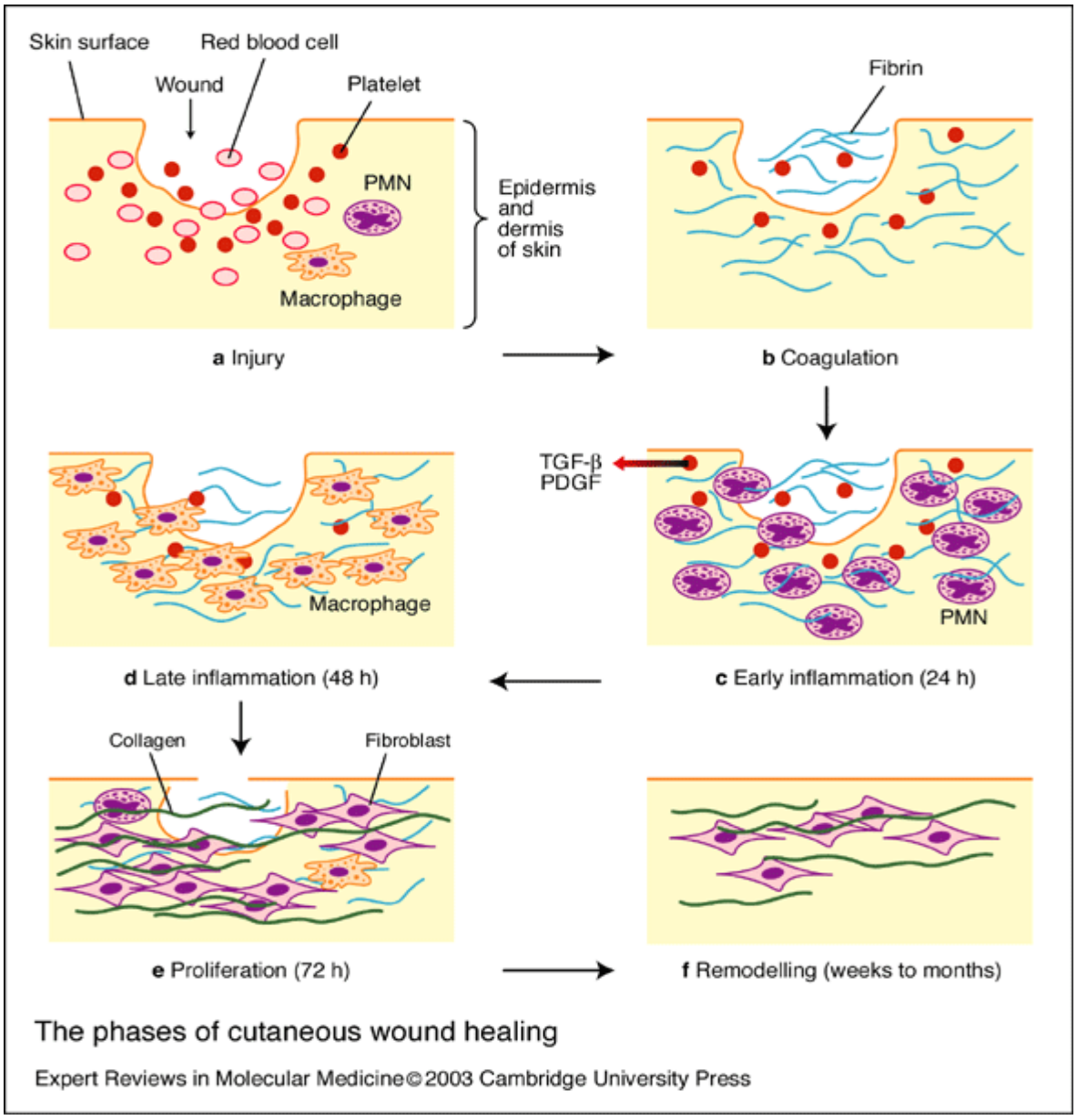


Figure 2.4 The phases of cutaneous wound healing. (a) Immediately following cutaneous injury. Platelets and plasma proteins are infiltrating to the wound. (b) Coagulation then occurs as platelets aggregate with fibrin. (c) Platelets release several factors, which attracts PMNs to the wound, signaling the beginning of inflammation. (d) After 48 h, PMNs and macrophages remove debris from the wound, release growth factors, and begin to reorganize the extracellular matrix. (e) The proliferation phase begins at about 72 h as fibroblasts, begin to synthesis collagen. (f) Collagen cross-linking and reorganization occur for months after injury in the remodeling phase of repair.

3.4 Types of wound healing (Kumar *et al*., 2005; Rubin and Farber, 1999)

Skin wounds are classically described to heal by primary or secondary intention. This distinction is based on the nature of the wound rather than the healing process itself.

3.4.1 Healing by primary intention (wounds with opposed edges)

The example of wound type is the healing of a clean, uninfected surgical incision. The incision causes minimum death of epithelial and connective tissue cells as well as disruption of epithelial basement membrane continuity. The incision space immediately fills with clotted blood containing fibrin and blood cells.

Within 24 hours, neutrophils infiltrate at the margins of the incision, to remove the fibrin clot. In 24 to 48 hours, the epithelial cells move from the wound edges (with little proliferation) along the incision margins of the dermis, depositing basement membrane components when they move and fuse in the midline under surface scab, producing a continuous but thin epithelial layer that closes the wound.

At day 3, the neutrophils are replaced by macrophages. Collagen fibers appear in the margin of the incision, but at first these are vertically oriented and do not bridge the incision. Granulation tissue progressively obtain in incision space and epithelial cell proliferate thickens the epidermal layer.

At day 5, granulation tissue fill in incision space. Collagen fibrils become more intense and begin to bridge the incision. The epidermis recovers its normal thickness and differentiation of epithelial cells to mature epidermal with surface keratinization.

During 2th week, there is continued deposition of collagen and proliferation of fibroblasts. At the wound site, leukocyte infiltrate, edema and vascularity, are decreased. At this time, the long process of blanching begins, performed by increased collagen deposition within the incision scar and regression of vascular channels.

For the 1st month, tensile strength closely parallels the collagen content of the wound. The epithelial cells on the surface divide and differentiate, there by restoring a multilayered epithelium.

After 1 to 3 month, as the granulation tissue is devascularized, the linear scar decreases in size and changes from red to white and the permanent scar is formed.

3.4.2 Healing by second intention (wound with separated edges)

Extensive tissues and cells loss or a simple failure to approximate the wound edges, as in surface wounds that create large defects. The degree of inflammation and the amount of granulation tissue are considerably greater in gouged wounds than in surgical incisions. Perhaps the feature that most clearly differentiates primary from secondary healing is the wound contraction, which occurs in large surface wounds. The wound contraction involves the formation of a network of actin-containing fibroblasts at the edge of the wound. The wound contraction decreases in the gap between the dermal edges of the wound. Whereas healing by primary intention is fast and leaves a small, often unapparent scar, while healing by secondary intention is slow and result in large of deforming scars.

3.5 Factors affecting wound healing (Kumar *et al*., 2005; Rubin and Farber, 1999; Stillman, 1998)

Wound healing is depended on two factors: local factors and systemic factors, related to the wound or to systems of the body.

3.5.1 Local factors

3.5.1.1 Infection

Wounds provide a portal of entry for microorganism. Infection is the single most important cause of delay in healing because it results in persistent tissue injury and inflammation and may result in large or deforming scars.

3.5.1.2 Size, location and type of wound

Small blunt wounds heal faster than larger ones. Wound in richly vascularized areas, such as the face, heals faster than those in poorly vascularized ones, such as the foot. In areas where the skin adheres to bony surfaces, as in injuries over the tibia, wound contraction and adequate apposition of the edges are difficult. A clean, aseptic wound produced by the surgeon is scalpel heals faster than a wound produced by blunt trauma, which exhibits abundant necrosis and irregular edges.

3.5.1.3 Mechanical factors

Early motion of wounds, can delay healing by compressing blood vessels and separating the edges of the wound. Particularly before tensile strength has been established and subjects a wound to persistent trauma.

3.5.1.4 Vascular supply

Wounds with impaired blood supply heal slowly. Disturbances to peripheral blood supply reduce tissue perfusion and limit the local supply of oxygen and nutrients required for repair. Reduced oxygen levels impair collagen synthesis. Oxygen is required as a cofactor and particularly for the hydroxylation steps, and epithelial growth and tissue resistance to infection.

3.5.1.5 Temperature fluctuations

Mitotic activity of cells occurs most rapidly at body temperatures. Extremes of temperatures prolong tissue repair. Low temperatures (a reduction of 2-3º F from normal core body temperature) cause extremity wounds to heal relatively slowly.

3.1.5.6 Foreign bodies

Foreign bodies such as unnecessary suture or fragments of dressing materials, steel and glass or fracture bone may cause tissue irritation which can prolong the inflammatory response and delay healing.

3.5.2 Systemic factors

3.5.2.1 Nutrition

Poor nutritional intake or lack of individual nutrients significantly alters many aspects of wound healing. Severe malnutrition impedes wound healing. For example, methionine is needed for proper healing. Zinc, a co-factor of several enzymes, promotes faster healing. Vitamin C is required for collagen synthesis and secretion. Vitamin C deficiency results in grossly deficient wound healing, with a lack of vascular proliferation and collagen deposition and cross-linking. Vitamin A deficiency impairs wound healing. Vitamin A increases the inflammatory response in wound healing by increasing the ability of lysosomal membranes. There is an increased influx of macrophages, with an increase in their activation and collagen synthesis.

3.5.2.2 Metabolic disorders

Metabolic status can change wound healing. Poorly controlled diabetes mellitus with ketoacidosis and hyperosmolarity is associated with delayed wound healing, as a consequence of microangiopathy that is a frequent feature of this disease, result in decreased cardiac output, poor peripheral perfusion and impaired polymorphonuclear leukocyte phagocytosis.

3.5.2.3 Hormones

Hormones, such as glucocorticoids, have well documented anti-inflammatory effects that influence various components of inflammation. These agents effect attributed to inhibition of collagen synthesis. However, these hormones also generally depress protein synthesis. Thyroid hormones, androgens, estrogens and growth hormone can also influence wound healing.

3.5.2.4 Drugs

Large dose or chronic usage of steroids reduce collagen synthesis and wound strength. Steroids inhibit the inflammatory phase of wound healing (angiogenesis, neutrophil and macrophage migration and fibroblast proliferation) and release of lysosomal enzymes. In addition to their effect on collagen synthesis, steroids also inhibit epithlialization and contraction and contribute to increased rates of wound infection, regardless of the time of administration. All chemotherapeutic antimetabolite drugs adversely affect wound healing by inhibiting early cell proliferation and wound DNA and protein synthesis, all of which are critical to successful repair.

4. Wound strength (Kumar *et al.*, 2005; Rubin and Farber, 1999; Stillman, 1998)

When sutures are removed, usually at the end of the first week, the wound strength of these wound are approximately 10% of wound strength of unwound skin. This rate of increase then slows at approximately the third month after the original incision and reaches a plateau at about 70% to 80% of tensile strength of

unwounded skin. The strength of heal wound depend on the deposition of an adequate extracellular matrix. The extracellular matrix is a stable complex of macromolecules that underlies epithelial cells and surrounds connective tissue cells. The extracellular matrix has five major components: collagens, basement membranes, elastic fibers, structural glycoproteins and proteoglycans.

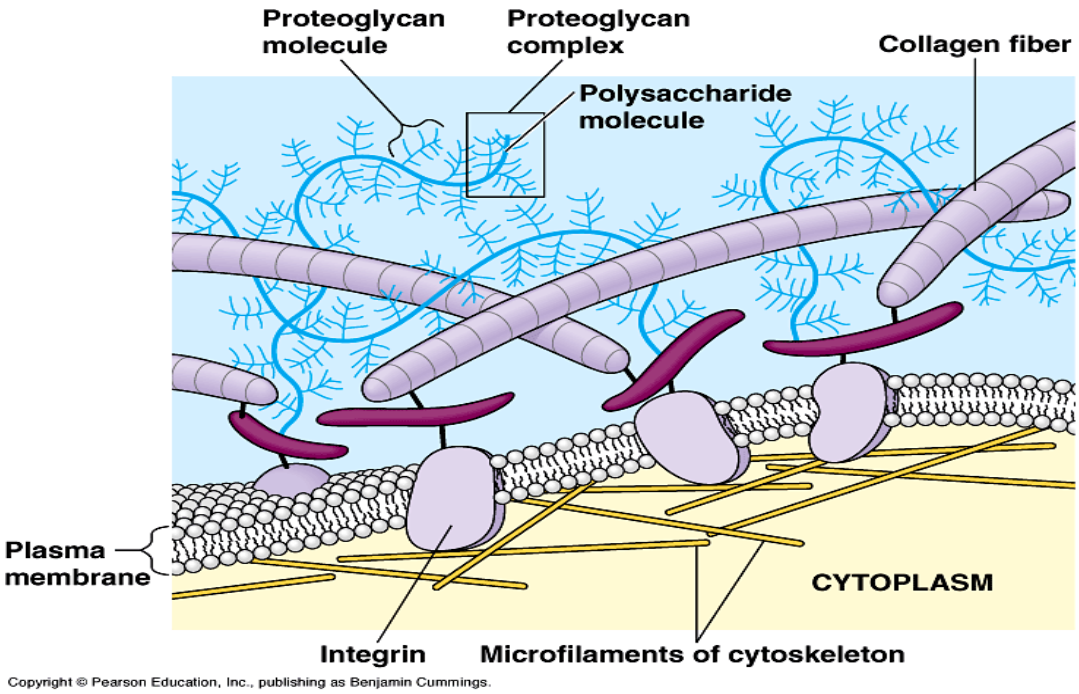


Figure 2.5 The extracellular matrix

4.1 Collagen

Collagen is the most abundant protein in the body, plays a critical role in the successful completion of wound healing. Its deposition, maturation and subsequent remodeling are essential to the function integrity of the wound. Although there are at least 18 types of collagen, but the main ones of interest of wound repair are type I and

type III. Type I collagen is the major component of extracellular matrix in skin. Type III collagen is also normally present in skin, it becomes more prominent and important during the repair process. Fibrillar collagen is synthesized from procollagen, a precursor molecular derived from preprocollagen, which is transcribed from collagen genes. After hydroxylation of proline and lysine residures and lysine gylcosylation, three procollagen chains align in phase to form the triple helix. Procollagen is secreted from the cell and cleaved by proteases to form the basic unit of the fibrils. Collagen fibril formation is associated with the oxidation of specific lysine and hydroxylysine residues by the extracellular enzyme lysyl oxidase. This results in cross-linking between the chains of adjacent molecules, thus stabilizing the array that is characteristic of collagen. Cross-linking is a major contributor to the tensile strength of collagen.

4.2 Elastic fiber

Whereas tensile strength is provided by member of the collagen family, the ability to recoil after transient stretching is provided by elastic fibers. Elastic fibers have two distinct components, a central amorphous core and a peripheral rim of microfibrils. Elastin have a 70 kd of glycoprotein, constitutes the central core of elastic fibers. Elastin is rich in glycerin and proline, but unlike collagen, it contains almost no hydroxylated amino acids. Elastin molecules are cross-linked to form an extensive network. Unlike most other proteins, elastin does not form definitive folds but rather oscillates between different states to form random coils.

4.3 Basement membrane

Basement membranes are complex structures at the interface between cells and stroma. They contain type IV collagen, laminin and other matrix components such as entactin and heparin sulfate proteoglycan. Basement membranes are synthesized by the

cells resting on them. The actual assembly of the various basement membranes components into a distinct entry is poorly understood, but it seems to occur extracellularly. Basement membranes are stable structures, which normally have a slow turnover. However, in certain situations such as wound healing basement membranes are rapidly degraded.

4.4 Structural glycoprotein

Glycoproteins whose major property is their ability to bind with other extracellular matrix component are structurally diverse. Glycoproteins consist of fibronectin, osteonectin and tenascin. Fibronectin exists in two major forms, plasma fibronectin and tissue fibronectin. Specific binding site in specialized domains of the fibronectin molecule allow it to bind avidly to collagens, proteoglycans, glycosaminoglycans, fibrinogen, fibrin, cell surfaces, bacteria and deoxyribonucleic acid (DNA). The varied binding properties of fibronectin permit it to connect cells with other conponents of the extracellular matrix, thereby integrating the tissue into a functional unit. Through the action of transglutaminases, fibronectin is covalently cross-linked with itself. This cross-linking is probably of great importance in the early phase of wound healing. Osteonectin is structural glycoprotein and bone associated, that binds to collagen type I and to hydroxylapatite. Tenascin is found in perichondrium, developing and mature tendons.

4.4 Proteoglycans

Proteoglycans are widely distributed in all extracellular matrices and also found in cell surfaces and in most biological fluids. The carbohydrate polymers were formerly termed mucopolysaccharides, but they are more properly referred to as glycosaminoglycans, because one of the sugar residues in the repeating disaccharide unit is always an amino sugar. Proteoglycans containing hydrated gels help maintain

tissue turgor. Their high charge density also allows them to act as selective filters. Proteoglycans participate in the organization of the extracellular matrix by binding to collagen fibers, elastic fibers and fibronectin. As organizers of the extracellular matrix, these molecules are deposited in the early phases of wound healing before collagen deposition becomes prominent.

5. Diabetes mellitus (LeRoith, Taylor and Olefsky, 2004)

Diabetes mellitus (DM) is a clinically and genetically heterogeneous group of disorders characterized by abnormally high levels of glucose in the blood. The hyperglycemia is due to deficiency of insulin secretion or to resistance of the body which are cells to the action of insulin or to a combination of these. Often there are also disturbances of carbohydrate, fat and protein metabolism. The diagnosis of diabetes mellitus is based on two fasting plasma glucose levels of 126 mg per dL or higher. Other options for diagnosis include two-hour postprandial plasma glucose (2hr PPG) readings of 200 mg per dL or higher after glucose load of 75 g (essentially, the criterion recommended by World Health Organization).

5.1 Classification of diabetes mellitus

The new classification system identifies the types of diabetes mellitus by THE AMERICAN DIABETES ASSOCIATION CLASSIFICATION SYSTEM in 1996 and 1997 as follow:

5.1.1 Type I diabetes mellitus

Caused by the β-cell destruction or often immune mediated that leads to loss of insulin secretion and absolute insulin deficiency. The etiologic agents that cause the autoimmune process and β-cell destruction are not well established. Also includes cases in which causes of the β-cell destruction are not understood. The onset is usually acute, developing over a period of a few days to weeks. Over 95% of persons with type 1 diabetes mellitus develop the disease before the age of 25, with an equal incidence in both sexes and an increased prevalence in the white population.

5.1.2 Type II diabetes mellitus

Type II diabetes mellitus caused by a combination of genetic and non-genetic factors that result in insulin resistance and insulin deficiency. The specific genes are not known but are under intense investigation. Non-genetic factors include increasing age, high caloric intake, overweight, central adiposity, sedentary lifestyle and low birth weight.

5.2 Diabetes and healing (Singer and Clark, 1999; Efron, Are and Park, 2005)

Diabetes mellitus is the best known of the metabolic disorders contributing to increased rates of wound infection and failure. Uncontrolled diabetes results in impaired angiogenesis, prolong inflammation, decreased synthesis of collagen, increased levels of proteinases and defective macrophage function. Additionally, the large and small vessel disease that is the hallmark of advanced diabetes contributes to local hypoxemia. Defects in granulocyte function, capillary ingrowths and fibroblast proliferation. Obesity, insulin resistance, hyperglycemia and diabetic renal failure all contribute significantly and independently to the impaired wound healing observed in diabetics.

CHAPTER III

MATERIALS AND METHODS

1. Materials

1.1 Chemicals

- Alcohol (Siribuncha, Thailand)
- Normal saline solution (Klean&Kare, Thailand)
- Streptozotocin (Sigma, USA)
- Formaline (Vidyaom CO., LTD, Thailand)
- Collagen assay kit (Biocolor, Northern Ireland, UK)
- Acetic acid (Sigma, USA)
- Paraffin (Tyco Heal Thcare Group LP., USA)
- Hematoxylin stain (Bio-Optica, Italia)
- Eosin stain (Bio-Optica, ITALIA)
- Citrate buffer (Sigma, USA)
- Pepsin (Fluka Bio Chemika, United States)
- Pentobarbital sodium (Ceva Animal Health LTD., Bangkok)
- Xyline (TJ Baker, USA)

1.2 Instruments

- Sterile surgical blade no. 11 (Feather Safety Razor CO., LTD., Japan)
- Electric clipper
- Black silk no. 3
- Tensiometer (EZ-Test Shimadzu Corporation, Japan)

- Biopsy punch (Tontarra, Germany)
- Verniercaliper
- Glucometer (Accu-Check advantage, USA)
- Glucose oxidase reagent strips (Accu-Check advantage, USA)
- Syringes
- Needles
- Cotton pads
- Light microscope

1.3 Experimental animals

A total of 120 male Sprague Dawley rats weighing 250-300 grams obtained from the National Laboratory Animal Center, Mahidol University, Salaya, NakhonPathom, Thailand were used in this study. The animals were caged in the air-conditioned room maintained temperature at 25 ± 1ºC with 12 hour alternate light/dark cycle and allowed free access to standard diet and water throughout the study. The rats were used after acclimatization to the laboratory environment for a 7 day period. The animals were randomizing divided into two groups of 60 animals each for non-diabetic and diabetic groups. In each group the animals were subdivided into five subgroups of twelve animals each for investigation on day 3 and day 7.

1.4 Test substances

ECa 233 was kindly supplied by Dr. Chamnan Patarapanich Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences Chulalongkorn University. They were prepared in a hydrogel (Jatt Inter Group) base to obtain ECa233 gel containing ECa233 in the different concentration as follow:

- 0.05% ECa233 Gel
- 0.1% ECa233 Gel
- 0.5% ECa233 Gel

2. Methods

2.1 Induction of diabetes

The rats were restricted diet and water for 12 hour before being induced to become diabetic by a single intravenous injection of streptozotocin (50 mg/kg body weight) prepared in 0.1 M citrate buffer, pH 4.0. Three days after the days of streptozotocin injection, fasting blood glucose levels was determined by glucometer. The animals with fasting blood glucose over 200 mg/dl were included in the experiment. Wounds were created on the 7^{th} day after the induction of diabetes (Chithra *et al.*, 1998).

2.2 Incision wound creation

The animals were anesthetized by an intraperitoneal injection of pentobarbital sodium 60 mg/kg body weight. The dorsal regions were shaved with an electric clipper and the surgical area was disinfected with 70% alcohol. On the right side of the dorsal surface of each animal, a 3 cm linear full thickness incision wound was made below the inferior edge of scapula using a no. 11 sterile surgical blade. Each incision wound was closed with 0.5 cm spaced interrupted no. 3 silk sutures. (Figure 3.1) Wounds were topically treated with 100 mg single application of test substances (gel base, 0.05%, 0.1% and 0.5% 0f ECa 233) for 3 and 7 days. Animals were allowed to recover and were housed individually in cages. They received diet and water ad libitum. On day 3 and 7 posts wounding the animals were sacrificed with an intraperitoneal injection of pentobarbital sodium 100 mg/kg body weight. Then the sutures were removed and

the heal wound of each animal was isolated for the determination of the wound collagen content, tensile strength and epidermal thickness by histological method (Figure 3.2). A total of 120 animals were included in this experiment and divided into non diabetic and diabetic groups of 60 animals each. The sixty animals of each group were divided into five groups of twelve animals each. In each group, the animals were subdivided into two groups of six animals each for evaluation on day 3 and 7 as described below (Jimenez and Rampy, 1999).

In both non-diabetic and diabetic groups: The animals were divided randomly into five groups of treatment as follows:

Group 1: Incision wound with no treatment (untreated)

Group 2: Incision wound with topical application of gel base once a day.

Group 3: Incision wound with topical application of 0.05% ECa 233 once a day.

Group 4: Incision wound with topical application of 0.1% ECa 233 once a day.

Group 5: Incision wound with topical application of 0.5% ECa 233 once a day.

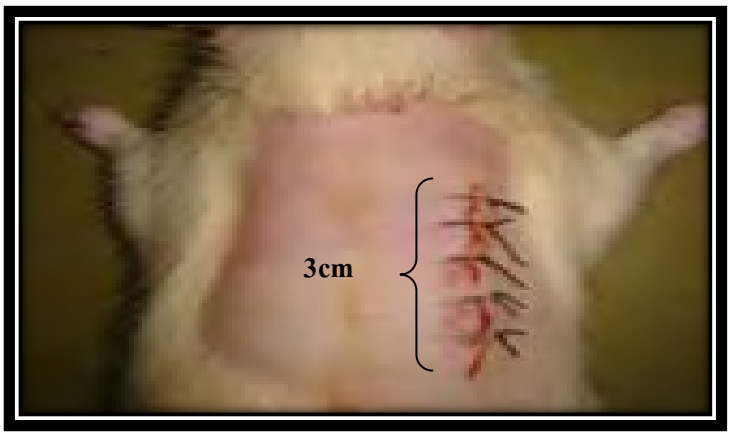


Figure 3.1 Rat skin incision was closed with interrupted sutures.

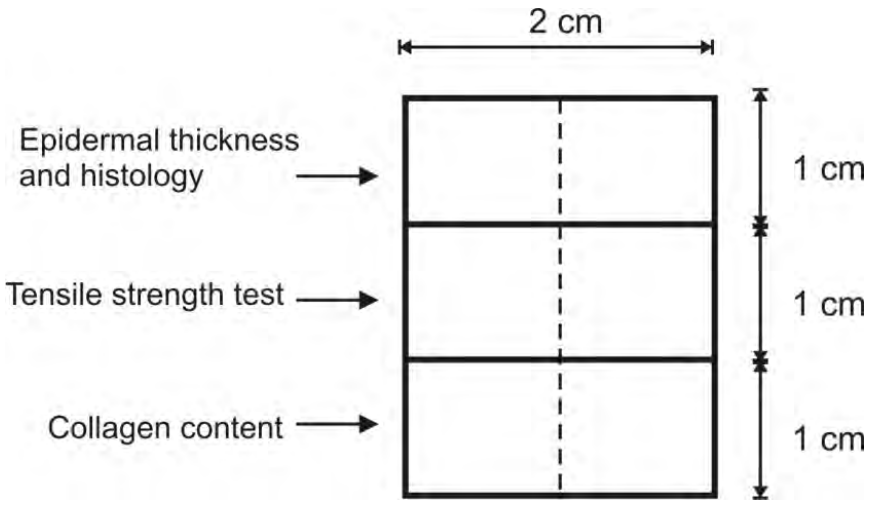


Figure 3.2 The preparation of tissue excised for further evaluations.

2.3 Assessment of wound healing

2.3.1 Tensile strength test

On day 3 and 7 posts wounding the animals were sacrificed with an overdose of pentobarbital sodium (100 mg/kg i.p.). Healing tissue was excised and sections were removed from each animal for tensile strength measurement using Tensiometer EZ- test (Figure 3.3). Strips of 1 cm width and 2 cm length were cut out from the excised tissue, and the fibrous tissue and subcutaneous tissue were trimmed out. Strips were loaded between two clamps of the tensiometer (jaw spaced of 0.5 cm). The total breaking load was measured in Newton and the tensile strength was calculated by the following equation (Shukla *et al.,* 1999) :

$$\text{Tensile strength } [N/cm^2] = \text{Breaking load } [N] / \text{Area } [cm^2]$$

$$\text{Area } [cm^2] = \text{Thickness } [cm] \times \text{Width } [cm]$$

Figure 3.3 The tensiometer

2.3.2 Biochemical analysis

The section of the wound 1 cm width and 2 cm length was excised from each animal and punched with 5 mm punch biopsy and were stored immediately at -80 ºC until determination. Collagen deposition was estimated by determining the total wound collagen content using the Sircol collagen assay kit (Biocolor, Northern Ireland, UK), a quantitative dry-binding assay which measure total collagen (type I-V) in tissue. The tissue biopsies were homogenized by pepsin (100 mg pepsin/gram wet tissue) in 0.5 ml of 0.5 M acetic acid. The samples were carried out for overnight at room temperature, and 1 ml of Sircol Dye reagent was added to each sample, and then mixed for 30 min. During this period the Sircol Dye will bind to soluble collagen, so that the collagen-dye complex will precipitate out of solution. Transfer the sample to microcentrifuge and spin at 12,000 x g for a 10 min. After centrifugation, the pellet was suspended in 1 ml of alkali reagent included in the kit for releasing Sircol Dye from the collagen-dye complex, and then the samples were mixed for 10 min by a vortex mixer. The samples were assessed colorimetrically at 540 nm by using microplate reader. Collagen standard solutions were used to construct a standard curve and calculated the collagen content of the test sample (Jimenez and Rampy, 1999).

2.3.3 Measurement of epidermal thickness and histological examination

The section of the wound 1 cm width and 2 cm length was excised from each animal. The tissue was preserved in the fresh fixative aqueous 10% neutral buffered solution of formaldehyde for 24 hrs then processed and embedded in paraffin. Serial sections of 10 µm were cut by using rotary microtome and stained with hematoxylin and eosin. The epidermal thickness was measured from stratum corneum layer to basal cell layer by using calibrated lens micrometer and the light microscope with x 4 and x 10 objective lens (Figure 3.4). To determine average epidermal

thicknesses surrounding the wound site, approximately 8 measures per specimen were randomly taken. The histology was assessed for morphology in wound area included fibroblast proliferation, epithelialization and collagen deposition. The light microscope with x 4 and x 10 objective lens was used (Jimenez and Rampy, 1999; Tang, Yin and Yang, 2007).

3. Statistical analysis

All results are expressed as mean ± S.E.M. and statistical analysis was performed by one-way analysis of variance followed by Duncan post hoc test. Data analysis was performed by using SPSS 16.0 statistical package programmed. $P<0.05$ was considered as statistically significant.

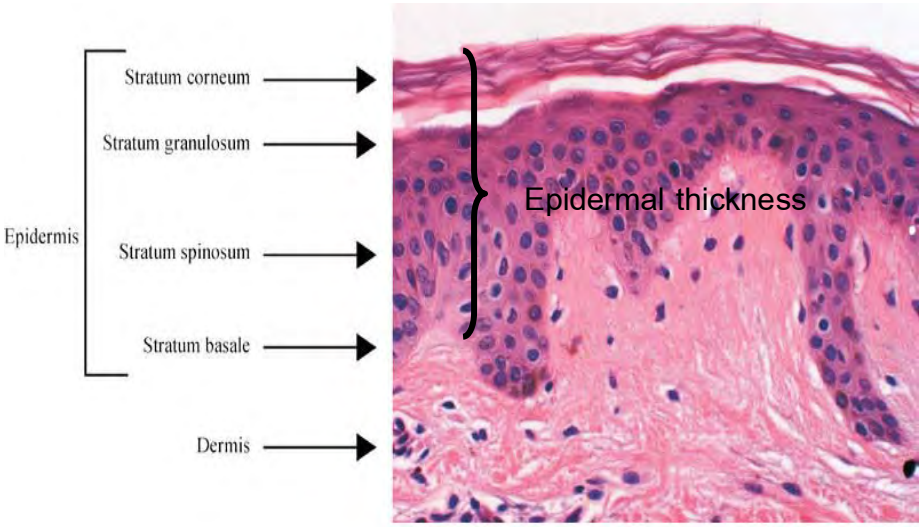


Figure 3.4 The epidermal thickness was measured from stratum corneum layer to basal cell layer.

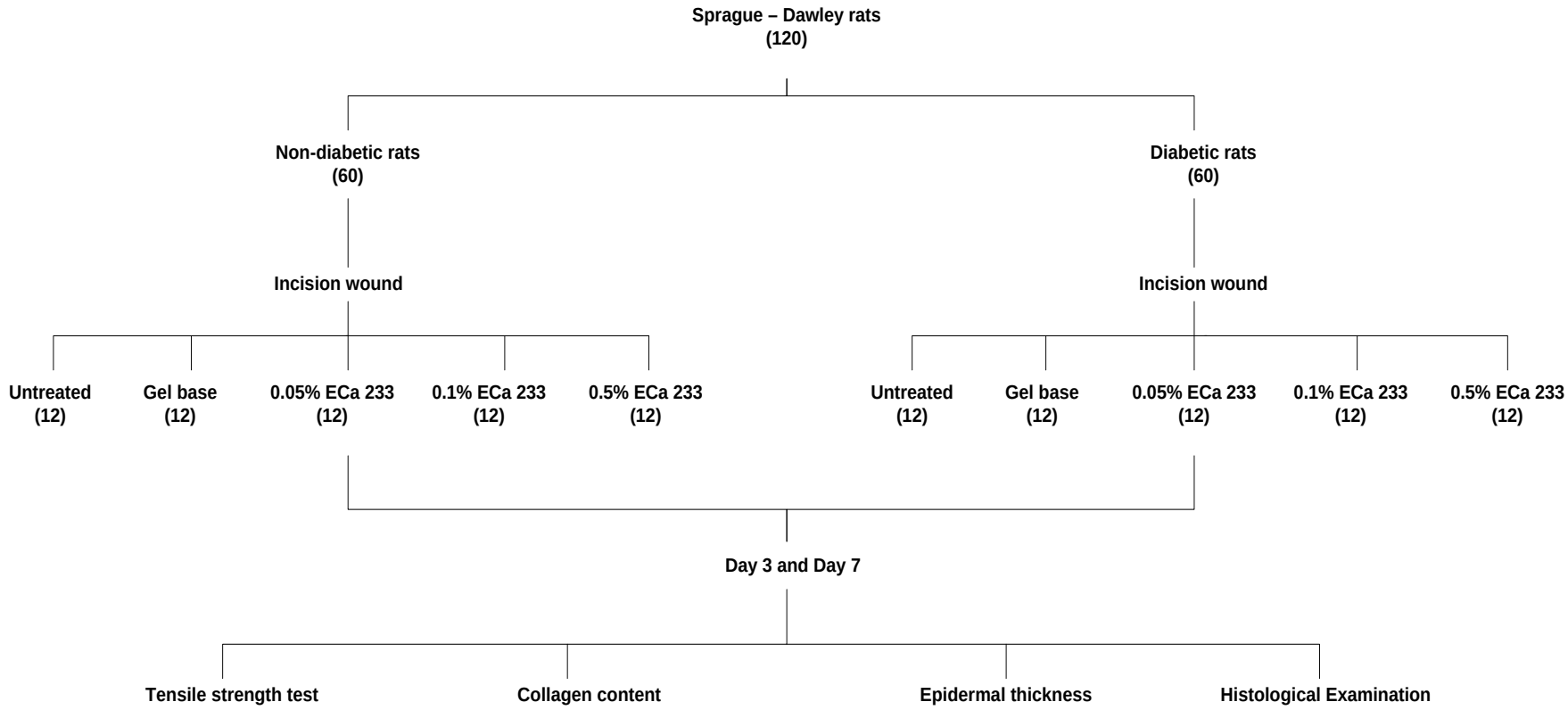


Figure 3.5 Diagram of experimental animal group

CHAPTER IV
RESULTS

1. Effects of standardized extract of *Centella asiatica* ECa 233 on wound healing in non-diabetic rats.

1.1 Tensile strength

Healing of incision wound was evaluated by a measurement of tensile strength, the force per unit of cross sectional area needed to break the wound. On day 3 post wounding, the tensile strength between untreated and gel base treated group which serve as a control group was not statistically significant different. In comparison to untreated and gel base treated groups, tensile strength in animal groups treated with 0.05% ECa 233 gel was statistically significant increased to 5.33 ± 1.78 N/cm^2 whereas it was 2.40 ± 0.41 N/cm^2 and 2.31 ± 0.39 N/cm^2 in untreated and gel base treated groups, respectively. However, tensile strength of animal groups treated with 0.1% and 0.5% ECa 233 gel was not statistically significant different from untreated and gel base treated groups. Furthermore, tensile strength on day 7 post wounding of animal groups treated with 0.05%, 0.1% and 0.5% ECa 233 gel were neither statistically significant different from untreated (14.58 ± 2.29 N/cm^2) nor gel base treated groups (17.72 ± 3.55 N/cm^2). (Figure 4.1, Table 4.1)

Groups	Tensile strength (N/cm^2)		Epidermal thickness (μm)		Collagen content (μg/mg tissue)	
	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
untreated	2.40 ± 0.41	14.58 ± 2.29	81.13 ± 7.23	137.17 ± 10.63	49.75 ± 2.79	59.67 ± 5.35
gel base	2.31 ± 0.39	17.72 ± 3.55	88.25 ± 9.66	148.71 ± 12.29	48.69 ± 3.32	63.98 ± 2.27
0.05% Eca 233	5.33 ± 1.78$^{*\#}$	16.14 ± 2.21	158.13 ± 11.38$^{*\#}$	159.67 ± 10.71	50.18 ± 2.96	58.03 ± 3.08
0.1% Eca 233	1.28 ± 0.43	18.41 ± 1.79	117.71 ± 6.33$^{*\#}$	155.96 ± 0.99	45.96 ± 3.08	70.37 ± 3.31
0.5% ECA 233	2.10 ± 0.58	19.21 ± 2.18	114.58 ± 8.25$^{*\#}$	158.92 ± 2.71	47.18 ± 2.32	61.36 ± 5.99

Table 4.1 Effects of ECa 233 on tensile strength, collagen content and epidermal thickness in non-diabetic rats. Results are presented as mean ± S.E.M. (n = 6) *$P \leq 0.05$ statistically significant different compare to untreated, #$P \leq 0.05$ statistically significant different compare to gel base treated.

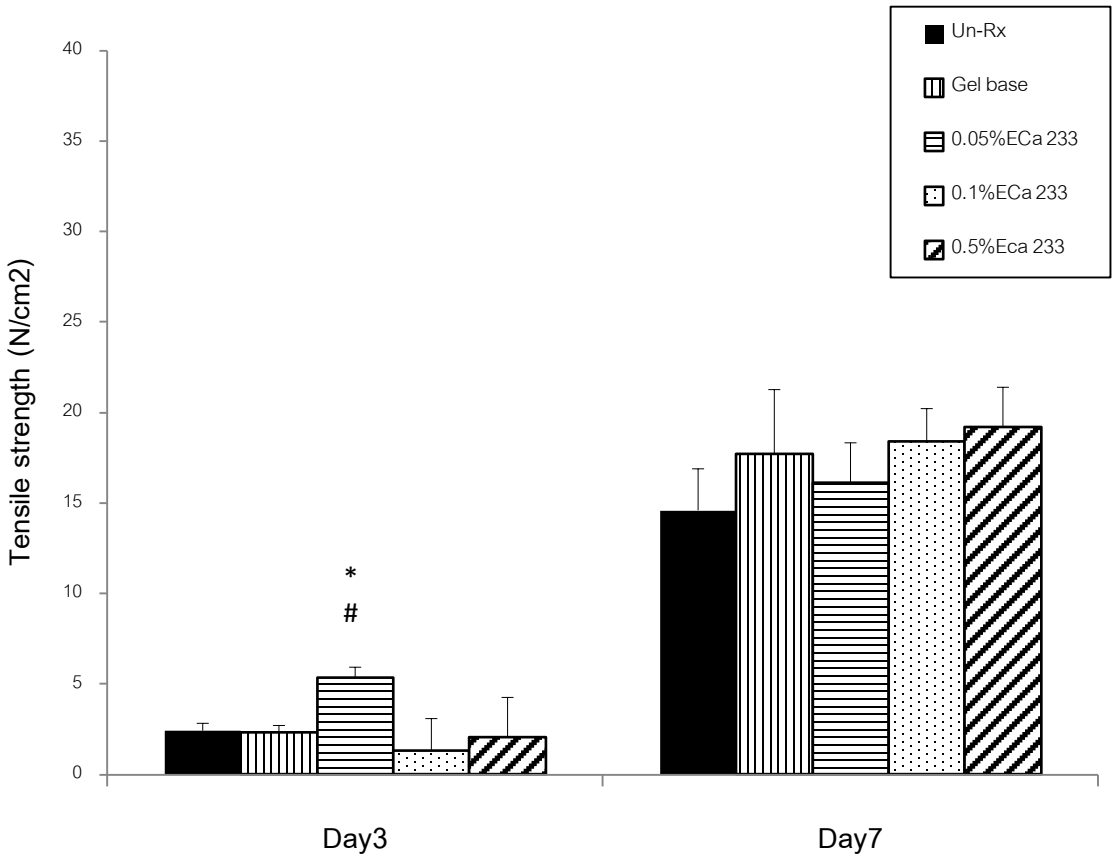


Figure 4.1 Effect of ECa 233 on tensile strength in incision wounds at the day 3 and 7 in non- diabetic rats. Results are presented as mean ± S.E.M. (n=6)

*$P \leq 0.05$ statistically significant different compare to untreated.

#$P \leq 0.05$ statistically significant different compare to gel base treated.

Un-Rx; incision wound with no treatment.

Gel base; incision wound treated with gel base.

0.05% ECa 233; incision wound treated with 0.05% ECa 233.

0.1% ECa 233; incision wound treated with 0.1% ECa 233.

0.5% ECa 233; incision wound treated with 0.5% ECa 233.

1.2 Epidermal thickness

Evaluation of epidermal thickness was measured from hematoxylin and eosin stained section of the excised tissue. On day 3 post wounding, it was found that epidermal thickness of untreated group (81.13 ± 7.23 μm) was not statistically significant different from those found in gel base treated group (88.25 ± 9.66 μm) which serve as a control groups. In comparison to untreated and gel base treated groups, epidermal thickness in animal groups treated with 0.05%, 0.1% and 0.5% ECa 233 gel was statistically significant increased to 158.13 ± 11.38 μm, 117.71 ± 6.33 μm, 114.58 ± 8.25 μm respectively whereas it was 81.13 ± 7.23 μm and 88.25 ± 9.66 μm in untreated and gel base treated groups, respectively. However, epidermal thickness on day 7 post wounding of animal groups treated with 0.05%, 0.1% and 0.5% ECa 233 gel was neither statistically significant different from untreated (137.17 ± 10.63 μm) nor gel base treated groups (148.71 ± 12.29 μm). (Figure 4.2, Table 4.1)

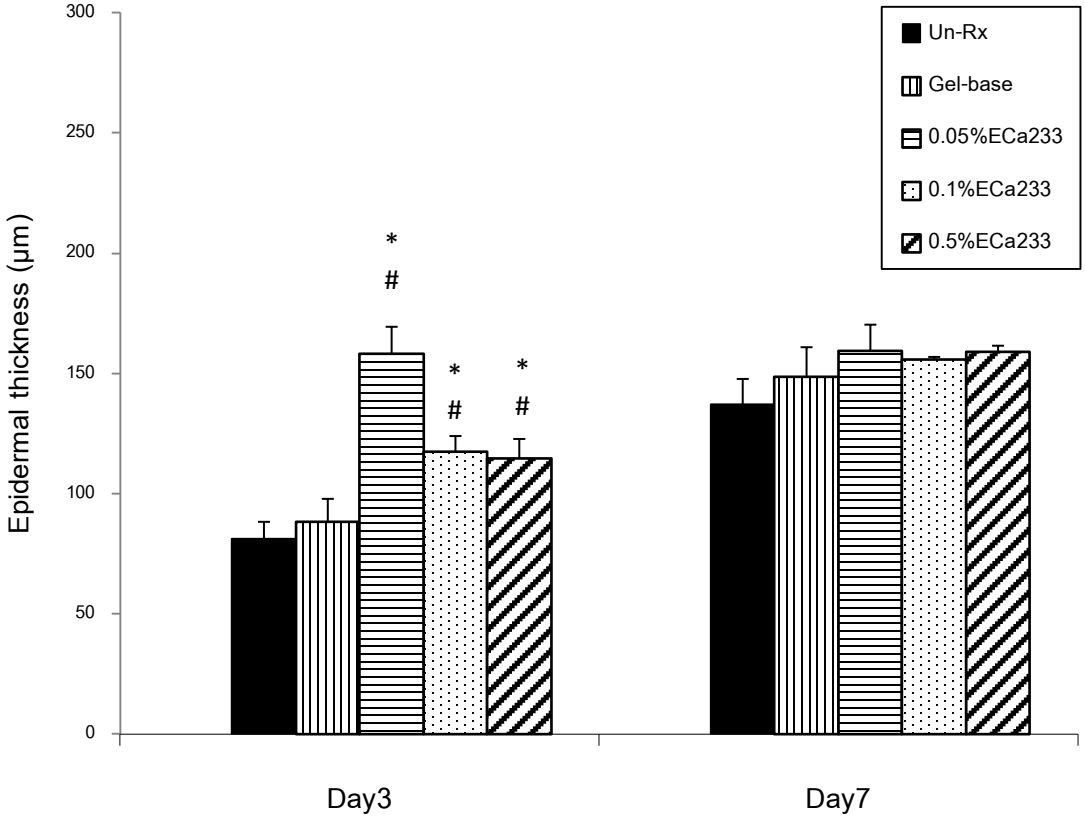


Figure 4.2 Effect of ECa 233 on epidermal thickness in incision wounds at the day 3 and 7 in non-diabetic rats. Results are presented as mean ± S.E.M. (n=6)

*$P \leq 0.05$ statistically significant different compare to untreated.

#$P \leq 0.05$ statistically significant different compare to gel base treated.

Un-Rx; incision wound with no treatment.

Gel base; incision wound treated with gel base.

0.05% ECa 233; incision wound treated with 0.05% ECa 233.

0.1% ECa 233; incision wound treated with 0.1% ECa 233.

0.5% ECa 233; incision wound treated with 0.5% ECa 233.

1.3 Collagen content

The wound collagen content was determined by Sircol collagen assay kit. Values are presented as micrograms total collagen (type I-V) per milligrams tissue wet weight. At day 3 post wounding collagen content of animal groups treated with 0.05%, 0.1% and 0.5% of ECa 233 was neither significantly different from those of untreated (49.75 ± 2.79 µg/mg) nor gel base groups (48.69 ± 3.32 µg/mg). Similar results were obtained at day 7 post wounding the wound collagen content of animal groups treated with 0.05%, 0.1% and 0.5% of ECa 233 was neither significantly different from those of untreated (59.67 ± 5.35 µg/mg) nor gel base groups (63.98 ± 2.27 µg/mg). (Figure 4.3, Table 4.1)

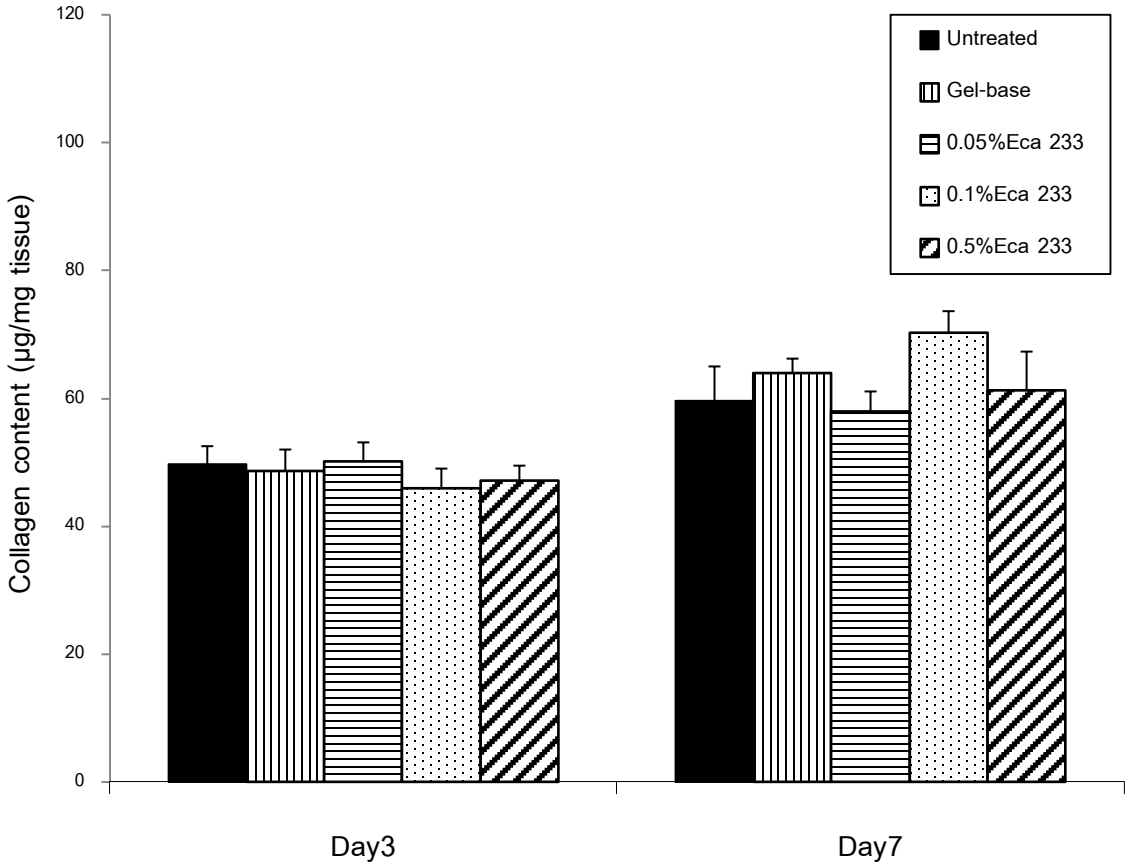


Figure 4.3 Effect of ECa 233 on collagen content in incision wounds at the day 3 and 7 in non- diabetic rats. Results are presented as mean ± S.E.M. (n=6)

*$P \leq 0.05$ statistically significant different compare to untreated.

#$P \leq 0.05$ statistically significant different compare to gel base treated.

Un-Rx; incision wound with no treatment.

Gel base; incision wound treated with gel base.

0.05% ECa 233; incision wound treated with 0.05% ECa 233.

0.1% ECa 233; incision wound treated with 0.1% ECa 233.

0.5% ECa 233; incision wound treated with 0.5% ECa 233.

1.4 Histological observation

The multiple sections used for histological examination of the tissue of the wounds area treated with ECa 233 (0.05%, 0.1% and 0.5%), gel base and untreated groups on day 3 and 7 are shown in Figure 4.4 and 4.5. The histological examination showed that the tissue regeneration was much greater and incision lines were closed in the skin wound treated with 0.05% ECa 233 on day 3 post wounding. The wound treated with different concentration of ECa 233 are better in re-epithelialization than untreated and gel base group. The collagen bundles, granulation tissue and fibroblast proliferation in wound area were seen. Densely pack granulation tissue were found along the incision line of rats treated with 0.05% ECa 233 while it was very dispersed on day 7. In contrast, the wound in untreated, gel base and different concentration of ECa 233 (0.05%, 0.1% and 0.5%) treated groups were rather similar on day 7. The collagen bundles were densely packed and fibroblast proliferated were found in all animal groups. The epithelialization covering the wound area was seen and mild neovasculalization was also seen.

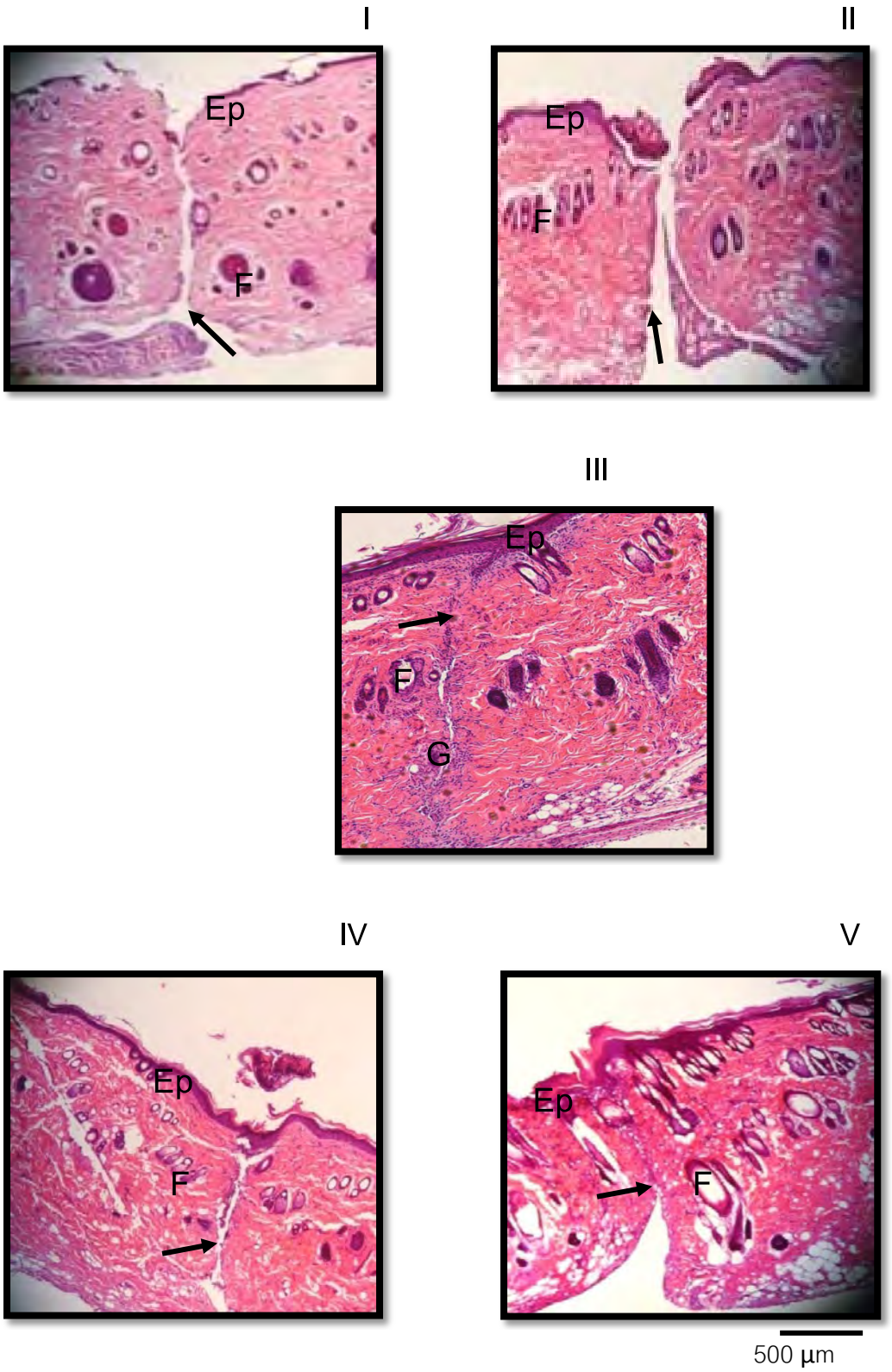


Figure 4.4 Histological examinations on day 3 post wounding. I: untreated, II: gel base treated, III: 0.05% ECa 233 treated, IV: 0.1% ECa 233 treated, V: 0.5% ECa 233 treated. Ep = Epidermis, F = Hair follicle, G = Granulation tissue, arrow indicated incision line.

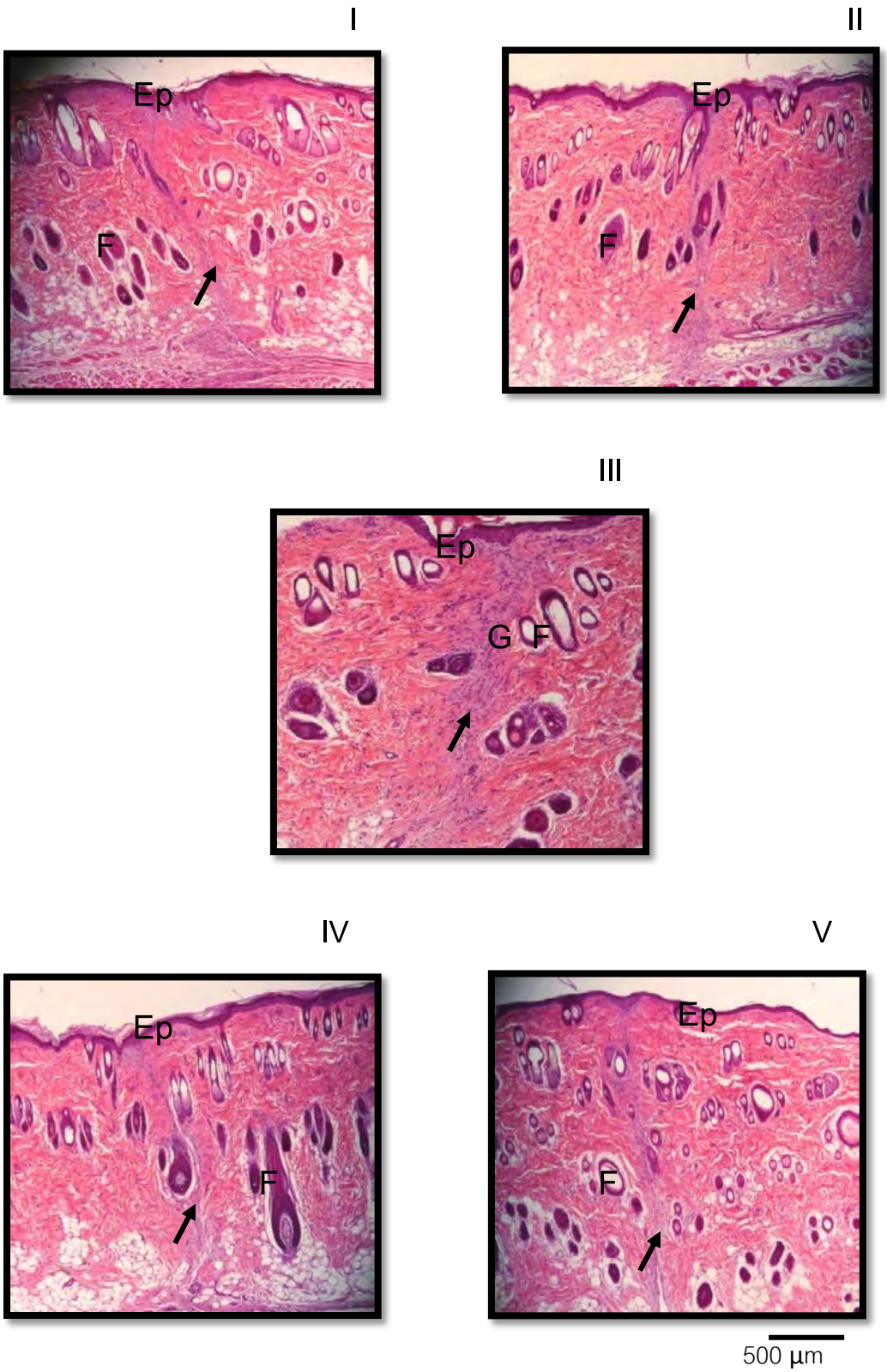


Figure 4.5 Histological examinations on day 7 post wounding. I: untreated, II: gel base treated, III: 0.05% ECa 233 treated, IV: 0.1% ECa 233 treated, V: 0.5% ECa 233 treated. Ep = Epidermis, F = Hair follicle, G = Granulation tissue, arrow indicated incision line.

2. **Effects of standardized extract of *Centella asiatica* ECa 233 on wound healing in diabetic rats.**

2.1 Tensile strength

Healing of incision wound was evaluated by a measurement of tensile strength, the force per unit of cross sectional area needed to break the wound. On day 3 post wounding, the tensile strength between untreated (2.19 ± 0.36 N/cm^2) and gel base treated group (2.96 ± 1.28 N/cm^2) which serve as a control group was not statistically significant different. In addition, tensile strength of animal groups treated with 0.05%, 0.1% and 0.5% ECa 233 gel was not statistically significant different from untreated and gel base treated groups. On day 7, while post wounding tensile strength of untreated and gel base treated group was not statistically significant different, tensile strength in animal groups treated with 0.05% ECa 233 gel was statistically significant increased to 12.77 ± 1.45 N/cm^2 whereas it was 7.55 ± 0.37 N/cm^2 and 6.98 ± 0.95 N/cm^2 in untreated and gel base treated groups, respectively. However tensile strength of animal groups treated with 0.1% and 0.5% ECa 233 gel was not statistically significant different from untreated and gel base treated groups. (Figure 4.6, Table 4.2)

Groups	Tensile strength (N/cm^2)		Epidermal thickness (μm)		Collagen content (μg/mg tissue)	
	Day 3	Day 7	Day 3	Day 7	Day 3	Day 7
untreated	2.19 ± 0.36	7.55 ± 0.37	84.54 ± 3.88	106.21 ± 3.36	42.65 ± 4.08	48.72± 0.98
gel base	2.96 ± 1.28	6.98 ± 0.95	84.75 ± 1.61	109.75 ± 1.74	40.52 ± 1.99	49.35 ± 4.99
0.05% Eca 233	2.67 ± 1.15	12.77 ± 1.45*#	90.83 ± 3.44	137.54 ± 1.92*#	46.26 ± 3.61	53.63 ± 3.29
0.1% Eca 233	1.76 ± 0.14	8.94 ± 1.89	89.42 ± 0.77	112.75 ± 2.36	48.23 ± 3.61	55.21 ± 3.19
0.5% ECA 233	2.00 ± 0.65	8.74 ± 1.95	89.63 ± 3.52	112.92 ± 3.22	50.77 ± 4.22	58.07 ± 0.88

Table 4.2 Effects of ECa 233 on tensile strength, collagen content and epidermal thickness in diabetic rats. Results are preseted as mean ± S.E.M. (n = 6) *$P \leq 0.05$ statistically significant different compare to untreated, #$P \leq 0.05$ statistically significant different compare to gel base treated.

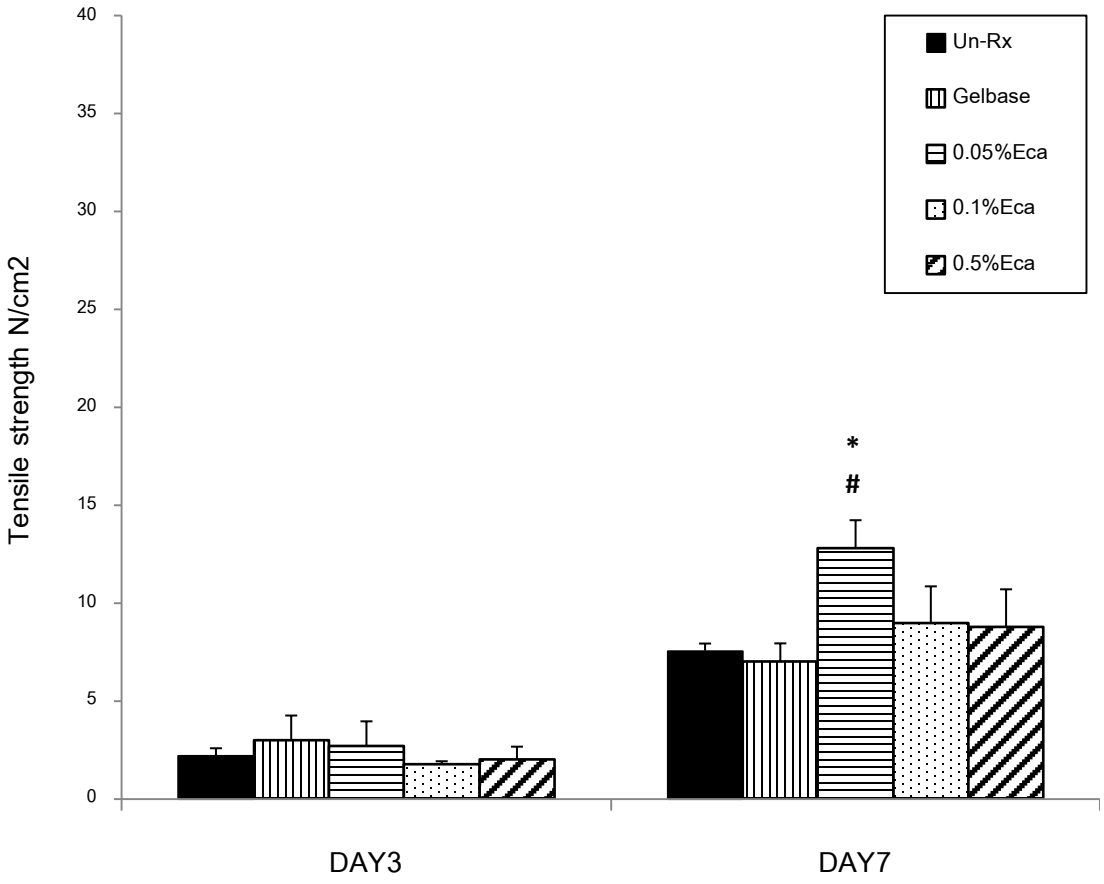


Figure 4.6 Effect of ECa 233 on tensile strength in incision wounds at the day 3 and 7 in diabetic rats. Results are presented as mean ± S.E.M. (n=6)

*$P \leq 0.05$ statistically significant different compare to untreated.

#$P \leq 0.05$ statistically significant different compare to gel base treated.

Un-Rx; incision wound with no treatment.

Gel base; incision wound treated with gel base.

0.05% ECa 233; incision wound treated with 0.05% ECa 233.

0.1% ECa 233; incision wound treated with 0.1% ECa 233.

0.5% ECa 233; incision wound treated with 0.5% ECa 233.

2.2 Epidermal thickness

Evaluation of epidermal thickness was measured from hematoxylin and eosin stained section of the excised tissue. On day 3 post wounding, it was found that epidermal thickness of untreated (84.54 ± 3.88 μm) was not statistically significant different from those found in gel base treated group (84.75 ± 1.61 μm) which serve as a control group. In addition, epidermal thickness of animal groups treated with 0.05%, 0.1% and 0.5% ECa 233 gel was neither statistically significant different from untreated nor gel base treated groups. However, epidermal thickness of untreated was not statistically significant different from those found in gel base treated group on day 7 post wounding. In comparison to untreated and gel base treated groups, epidermal thickness in animal groups treated with 0.05% ECa 233 gel was statistically significant increased to 137.54 ± 1.92 μm whereas it was 106.21 ± 3.36 μm and 109.75 ± 1.74 μm in untreated and gel base treated groups, respectively. In addition, epidermal thickness of animal groups treated with 0.1% and 0.5% ECa 233 gel was neither statistically significant different from untreated nor gel base treated groups. (Figure 4.7, Table 4.2)

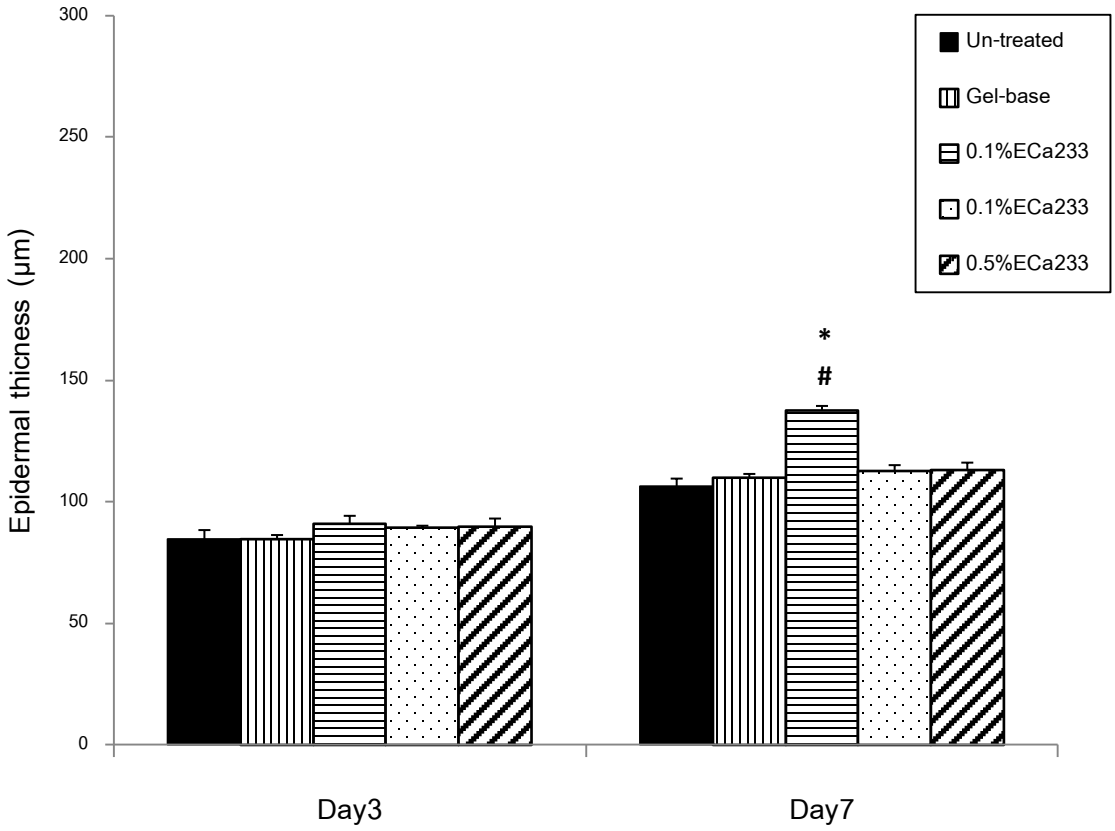


Figure 4.7 Effect of ECa 233 on epidermal thickness in incision wounds at the day 3 and 7 in diabetic rats. Results are presented as mean ± S.E.M. (n=6)

*$P \leq 0.05$ statistically significant different compare to untreated.

#$P \leq 0.05$ statistically significant different compare to gel base treated.

Un-Rx; incision wound with no treatment.

Gel base; incision wound treated with gel base.

0.05% ECa 233; incision wound treated with 0.05% ECa 233.

0.1% ECa 233; incision wound treated with 0.1% ECa 233.

0.5% ECa 233; incision wound treated with 0.5% ECa 233.

2.3 Collagen content

The wound collagen content was determined by Sircol collagen assay kit. Values are presented as micrograms total collagen (type I-V) per milligrams tissue wet weight. At day 3 post wounding collagen content of animal groups treated with 0.05%, 0.1% and 0.5% of ECa 233 was neither significantly different from those of untreated (42.65 ± 4.08 µg/mg) nor gel base groups (40.52 ± 1.99 µg/mg). Similar results were obtained at day 7 post wounding the wound collagen content of animal groups treated with 0.05%, 0.1% and 0.5% of ECa 233 was neither significantly different from those of untreated (48.72 ± 0.98 µg/mg) nor gel base groups (49.35 ± 4.99 µg/mg). (Figure 4.8, Table 4.2)

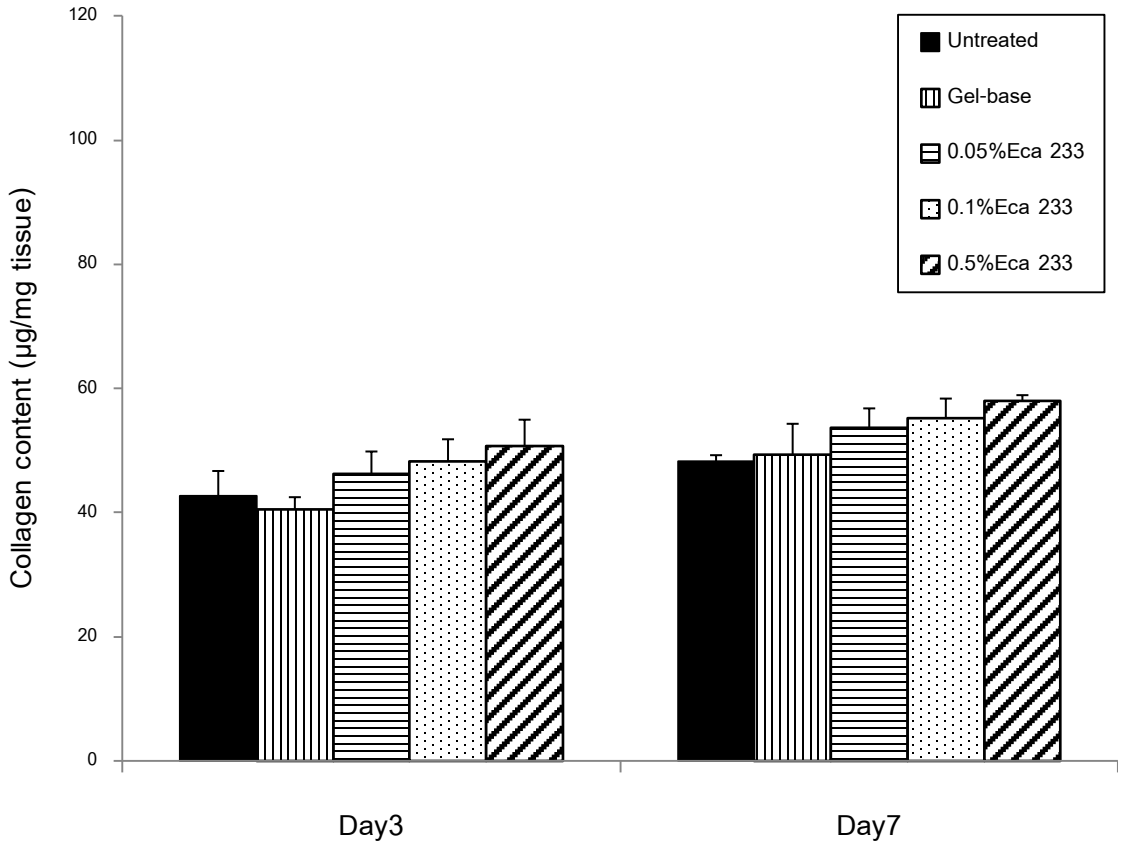


Figure 4.8 Effect of ECa 233 on collagen content in incision wounds at the day 3 and 7 in diabetic rats. Results are presented as mean ± S.E.M. (n=6)

*$P \leq 0.05$ statistically significant different compare to untreated.

#$P \leq 0.05$ statistically significant different compare to gel base treated.

Un-Rx; incision wound with no treatment.

Gel base; incision wound treated with gel base.

0.05% ECa 233; incision wound treated with 0.05% ECa 233.

0.1% ECa 233; incision wound treated with 0.1% ECa 233.

0.5% ECa 233; incision wound treated with 0.5% ECa 233.

2.4 Histological observation

The multiple sections used for histological examination of the tissue of the wounds area treated with ECa 233 (0.05%, 0.1% and 0.5%), gel base and untreated groups on day 3 and 7 are shown in Figure 4.9 and 4.10. The wound in untreated, gel base and different concentration of ECa 233 (0.05%, 0.1% and 0.5%) treated groups was rather similar on day 3. The healing union was not perfect in re-epithelialization, the collagen bundles were loosely packed and fibroblast proliferation was a moderate and absence of neovasculalization. The wound treated with 0.05% ECa 233 showed that the tissue healing was much greater in the re-epithelialization, fibroblast proliferation accumulation of granulation tissue and close of incision line. The collagen bundles that were densely packed was seen in all animal treated groups but absent in neovasculalization on day 7.

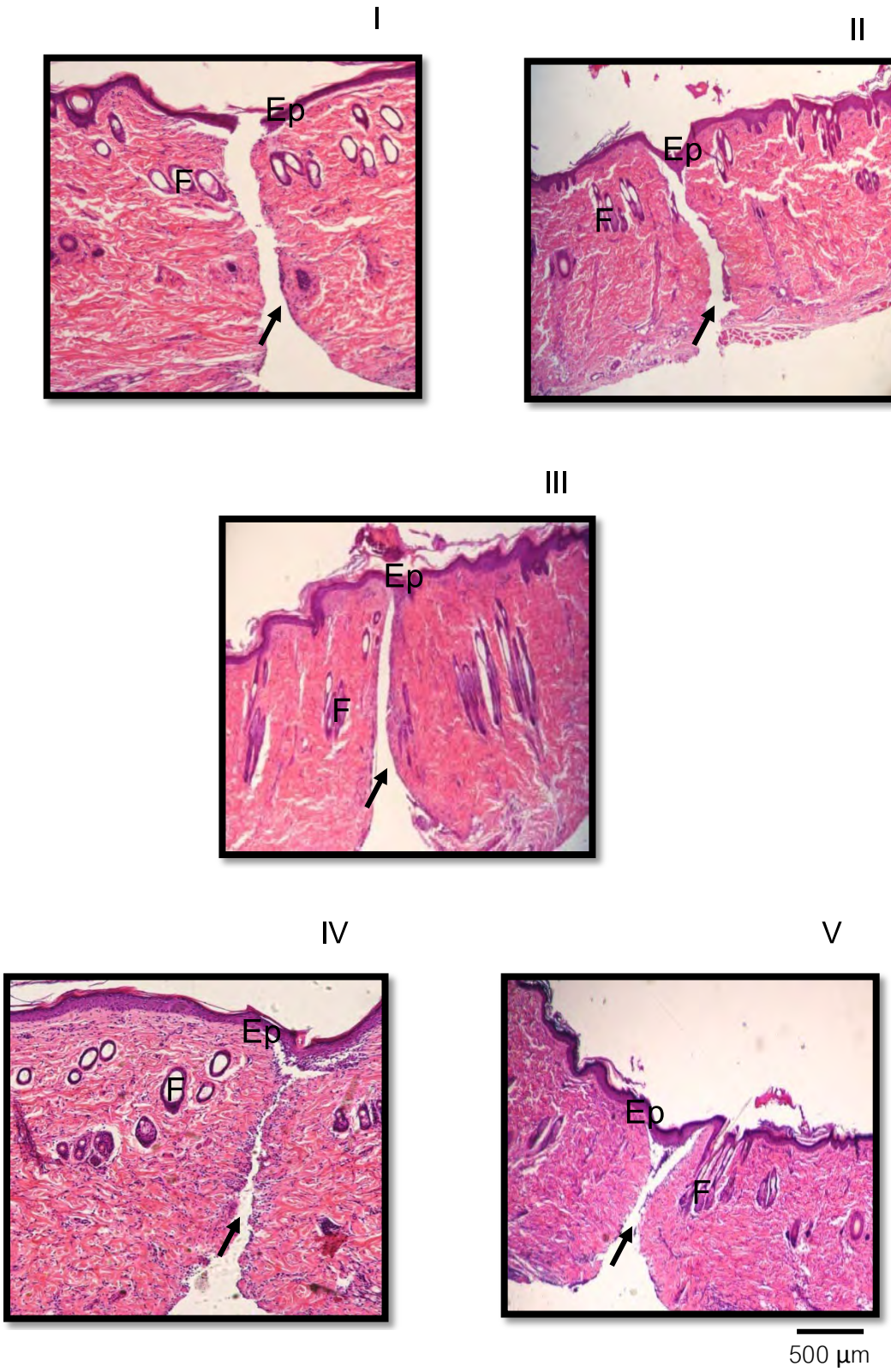


Figure 4.9 Histological examinations on day 3 post wounding. I: untreated, II: gel base treated, III: 0.05% ECa 233 treated, IV: 0.1% ECa 233 treated, V: 0.5% ECa 233 treated. Ep = Epidermis, F = Hair follicle, arrow indicated incision line.

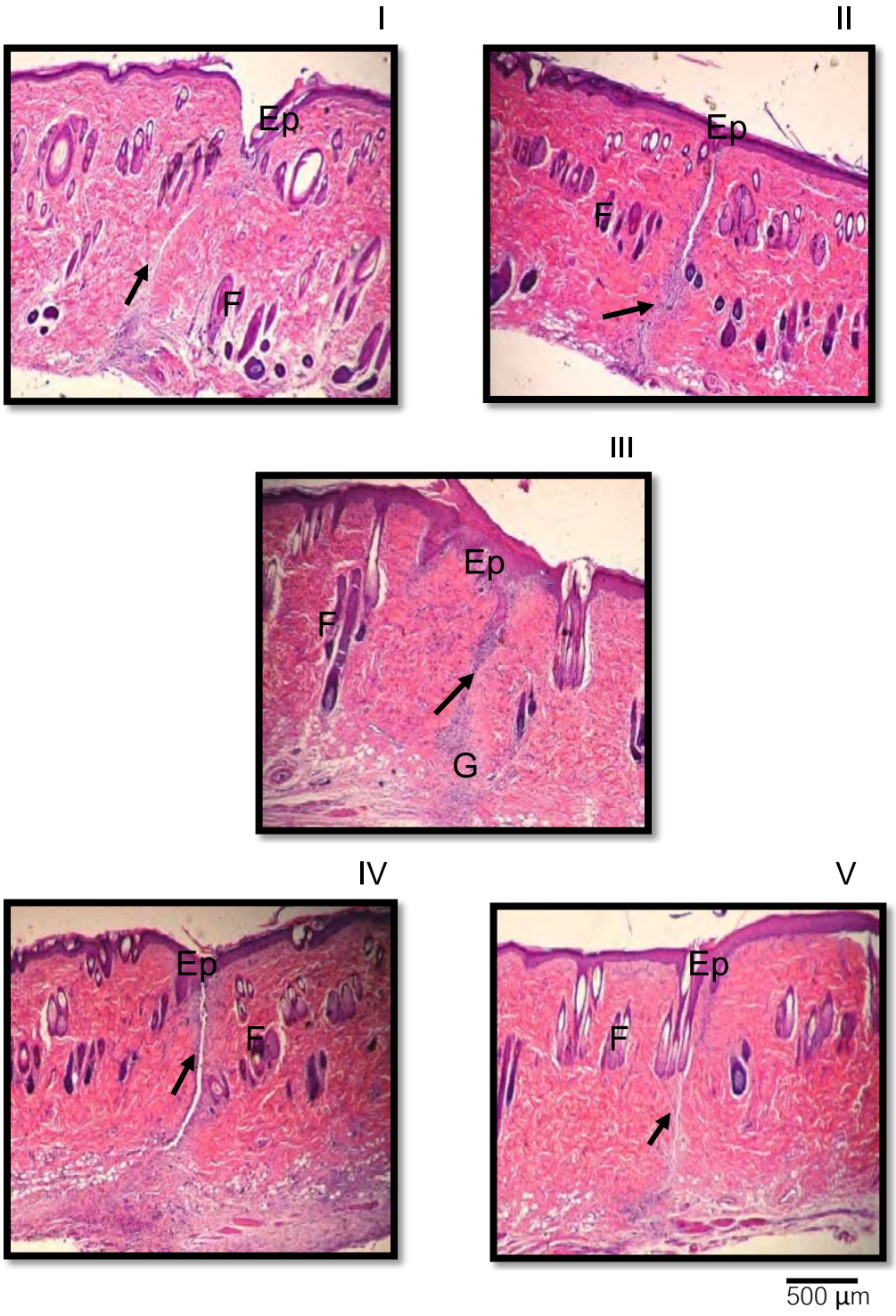


Figure 4.10 Histological examinations on day 7 post wounding. I: untreated, II: gel base treated, III: 0.05% ECa 233 treated, IV: 0.1% ECa 233 treated, V: 0.5% ECa 233 treated. Ep = Epidermis, F = Hair follicle, G = Granulation tissue, arrow indicated incision line.

CHAPTER V

DISCUSSION AND CONCLUSION

A wound is defined as a loss or breaking of cellular and anatomic of functional continuity of tissues. Healing of wound is a biological process that is initiated by trauma and often terminated by scar formation (Prassad and Dorle, 2006). Wound healing process consists of different phases and overlapping pattern of event including hemostasis, which leads to an inflammatory cell cascade invaded the wound site within a few hours after injury. Neutrophils arrived first, following by monocytes (24 to 48 hrs), macrophages and polymorphonuclear cells release important growth factors and cytokines, initiating granulation tissue formation. Furthermore, appearance of the fibroblasts (24 hrs) and collagen fibrils (3 days) near the cell surface was evident in the linear incision of the skin (Todorovic *et al.,* 2008).

Efforts are being made all over the world to discover agents that can promote healing. A number of studies indicate that plant products are potential agents for wound healing and largely preferred because of their widespread availability, absence of unwanted side effects and their effectiveness. Previous studies have shown that asiaticoside, isolated from *Centella asiatica*, could promote fibroblast proliferation and extracellular matrix synthesis in wound healing (Lu *et al.,* 2004). Moreover, the topical application and oral route of asiaticoside increases hydroxyproline, tensile strength, collagen content and re-epithelialization on punch wound model (Shukla *et al.,* 1999).

The present study demonstrated the effects of ECa 233, standardized extract of *Centella asiatica*, on incision wound healing in non-diabetic and diabetic rats. Linear incision wound healing models are used for wound healing bioassays because it

represents a true surgical wound that could be reproducibly analyzed in a non-subjective and highly controlled manner (Beloz *et al*., 2003). The progress of incision wound healing was assessed by measurement of tensile strength, histology, thickness of epidermis and total collagen content. One of the most important factors in the healing of wound is the stimulation of wound strength (Jimenez and Rampy, 1999). The tensile strength is a necessary parameter in determining the pharmacological effects of potential wound healing agents on incision wound. In non-diabetic rats, the result showed that tensile strength, defined as the force per unit of cross sectional area needed to break the wound, in groups receiving topical application of 0.05% ECa 233 was significantly increased higher than those of untreated and gel base treated groups on day 3 post wounding. These data suggest that 0.05% ECa 233 rapidly increases the strength of incision wounds in the early phases of healing.

Collagen is the predominant extracellular protein in the granulation tissue of a healing wound. They are synthesized by fibroblast begins within 3 to 5 days after injury and continues for several weeks which depending on the size of wound (Kumar *et al.,* 2005). The collagen molecules synthesis are decreased in the wound site and became cross-linked to form fibers result in increased strength and resistance to enzymatic degradation (Chithra *et al*., 1998). The strength of wounds ultimately depends on the deposition of an adequate extracellular matrix which is composed of five major components such as collagens, basement membranes, structural glycoproteins, elastic fibers and proteoglycans (Rubin and Farber, 1999). Although collagen content which is associated to tensile strength was not significantly increases in the present studies, it is likely that beneficial effect of ECa 233 on wound healing could be explained by its effect on the production of other extracellular matrix protein such as basement membranes, structural glycoproteins, elastic fibers and proteoglycans than stimulation of collagen synthesis. In addition, ECa 233 may exert effect on cross-linking of collagen which is correlated with tensile strength. Accordingly, histological evaluation showed that there

was an accumulation of granulation tissue in wound area of 0.05% ECa 233 treated groups indicating accelerated proliferative phase of wound healing.

Epithelialization of an incision wound involves the migration of cells at the wound edges from one side of the incision to the other within 24-48 hrs after injury (Kumar *et al.,* 2005). Base on the results observed in histological studies, epidermal thickness of non-diabetic rats treated with 0.05%, 0.1% and 0.5% of ECa 233 was found to be significantly higher than those of untreated and gel base treated groups on day 3 post wounding. The stimuli for migration and proliferation of epidermal cells were controlled by epidermal growth factor, transforming growth factor α and keratinocyte growth factor (Singer and Clark, 1999). Therefore, it is likely that ECa 233 migth stimulate the expression of some growth factors. Blood supply is factors that influence wound healing. Wounds with impaired blood supply heal slowly and disturbances to peripheral blood supply reduce tissue perfusion and limit the local supply of oxygen and nutrients required for repair. Recent studies found that ECa 233 significantly increase blood supply to wound area in burn wound model (Wannarat *et al.,* 2009) and that could be at least, in part underlie acceleration of wound healing observed in incision wound model in the present study.

No significant difference in tensile strength, total collagen content and epidermal thickness was observed in all animal groups of non-diabetic on day 7 post wounding. The finding in histological studies obviously demonstrated that the healing union was perfect in terms of epithelialization and fibroblast proliferation. Furthermore collagen bundles were densely packed, suggesting that the remodeling phase of wound healing initiated by a formation of granulation tissue, has converted to a stable extracellular matrix.

One of the leading causes of impair wound healing is diabetes mellitus. In diabetic patients, a cutaneosus wound often exhibits prolonged inflammation, impaired neovascularization, decreased synthesis of collagen and defective macrophage function resulting in delay wound healing and infection (Singer and Clark, 1999). To investigate the pharmacological effect of ECa 233 on the incision wound of diabetes mellitus, diabetes mellitus model of rats was induced by an injection of STZ which is widely used as chemical inducer of insulin-dependent diabetes mellitus. STZ has been shown to produce free radicals in the body that specifically cut DNA chains in the pancreatic β cell, resulting in disorder of pancreatic β cell function (Li *et al*., 2008). Recent study demonstrated that STZ induced diabetes mellitus in rats resulted in a decrease in blood flow of the wound (Wannarat *et al.,* 2009). The wound with impair blood supply thus caused a delay in wound healing. The present study demonstrated that diabetic mellitus caused a delay in wound healing induced by STZ in both placebo treated and ECa 233 treated groups. The beneficial effect of ECa 233 on wound healing demonstrated in non-diabetic rats at day 3 was not detected in diabetic rats. The tensile strength, total collagen content and epidermal thickness of different concentrations of ECa 233 treated group were not significantly different from untreated and gel base treated groups on day 3 post wounding. However on the day 7 post wounding, the tensile strength and epidermal thickness except collagen content were significantly increased in 0.05% of ECa 233 treated group. The data suggest that 0.05% of ECa 233 could improve a wound healing in diabetic rats, however, with lower rate than that in non-diabetic rats. Excessive oxidative stress generally found in diabetes mellitus could possibly accounted for such a delay in wound healing (Bonnefont *et al.,* 2004). Previous report demonstrated that asiaticoside significantly increased enzymatic and non-enzymatic antioxidants (superoxide dismutase, catalase, glutathione peroxidase, vitamin E and ascorbic acid) and decreased in lipid peroxide level (Shukla *et al.,* 1999) and that could be at least, in part underlie acceleration of wound healing observed in diabetic rats in the present study.

In conclusion, this study demonstrated that a single daily topical application of 0.05% ECa 233 shows significant acceleration of wound healing activity on incision wound in non-diabetic as well as in diabetic rats. Increases in both tensile strength and epidermal thickness could possibly be accounted by stimulation of other component of extracellular matrix and some growth factor than collagen. Some other possible mechanisms of standardized extract of *Centella asiatica* ECa 233 on wound healing should be further investigated.

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APPENDICES

Effect of ECa 233 on tensile strength of incision wound in non-diabetic rats

Groups	Tensile strength (N/cm2)	
	Day 3	Day 7
untreated	2.40 ± 0.41	14.58 ± 2.29
gel base	2.31 ± 0.39	17.72 ± 3.55
0.05% ECa 233	5.33 ± 1.78 * #	16.14 ± 2.21
0.1% Eca 233	1.28 ± 0.43	18.41 ± 1.79
0.5% Eca 233	2.10 ± 0.58	19.21 ± 2.18

Table 1: The effect of different concentration of ECa 233 on tensile strength in non-diabetic rats. The values are expressed as the mean ± S.E.M. (n=6). Statistical analyses were performed by one-way ANOVA and Duncan for comparison. A significant value of p less than 0.05 was considered as statistically significant.

*Significantly different from value in untreated group.

Significantly different from value in gel base group.

Effect of ECa 233 on epidermal thickness of incision wound in non-diabetic rats

Groups	Epidermal thickness (μm)	
	Day 3	Day 7
untreated	81.13 ± 7.23	137.17 ± 10.63
gel base	88.25 ± 9.66	148.71 ± 12.29
0.05% ECa 233	158.13 ± 11.38*#	159.67 ± 10.71
0.1% Eca 233	117.71 ± 6.33*#	155.96 ± 0.99
0.5% Eca 233	114.58 ± 8.25*#	158.92 ± 2.71

Table 2 : The effect of different concentration of ECa 233 on epidermal thickness in non-diabetic rats. The values are expressed as the mean ± S.E.M. (n=6). Statistical analyses were performed by one-way ANOVA and Duncan for comparison. A significant value of p less than 0.05 was considered as statistically significant.

* Significantly different from value in untreated group.

Significantly different from value in gel base group.

Effect of ECa 233 on collagen content of incision wound in non-diabetic rats

Groups	Collagen content (µg/mg tissue)	
	Day 3	Day 7
untreated	49.75 ± 2.79	59.67 ± 5.35
gel base	48.69 ± 3.32	63.98 ± 2.27
0.05% ECa 233	50.18 ± 2.96	58.03 ± 3.08
0.1% Eca 233	45.96 ± 3.08	70.37 ± 3.31
0.5% Eca 233	47.18 ± 2.32	61.36 ± 5.99

Table 3 : The effect of different concentration of ECa 233 on collagen content in non-diabetic rats. The values are expressed as the mean ± S.E.M. (n=6). Statistical analyses were performed by one-way ANOVA and Duncan for comparison. A significant value of p less than 0.05 was considered as statistically significant.

* Significantly different from value in untreated group.

Significantly different from value in gel base group.

Effect of ECa 233 on tensile strength of incision wound in diabetic rats

Groups	Tensile strength (N/cm2)	
	Day 3	Day 7
untreated	2.19 ± 0.36	7.55 ± 0.37
gel base	2.96 ± 1.28	6.98 ± 0.95
0.05% ECa 233	2.67 ± 1.15	12.77 ± 1.45 * #
0.1% Eca 233	1.76 ± 0.14	8.94 ± 1.89
0.5% Eca 233	2.00 ± 0.65	8.74 ± 1.95

Table 4 : The effect of different concentration of ECa 233 on tensile strength in diabetic rats. The values are expressed as the mean ± S.E.M. (n=6). Statistical analyses were performed by one-way ANOVA and Duncan for comparison. A significant value of p less than 0.05 was considered as statistically significant.

*Significantly different from value in untreated group.

Significantly different from value in gel base group.

Effect of ECa 233 epidermal thickness on of incision wound in diabetic rats

Groups	Epidermal thickness (μm)	
	Day 3	Day 7
untreated	84.54 ± 3.88	106.21 ± 3.36
gel base	84.75 ± 1.61	109.75 ± 1.74
0.05% ECa 233	90.83 ± 3.44	137.54 ± 1.92 * #
0.1% Eca 233	89.42 ± 0.77	112.75 ± 2.36
0.5% Eca 233	89.63 ± 3.52	112.92 ± 3.22

Table 5 : The effect of different concentration of ECa 233 on epidermal thickness in diabetic rats. The values are expressed as the mean ± S.E.M. (n=6). Statistical analyses were performed by one-way ANOVA and Duncan for comparison. A significant value of p less than 0.05 was considered as statistically significant.

* Significantly different from value in untreated group.

Significantly different from value in gel base group.

Effect of ECa 233 on collagen content of incision wound in diabetic rats

Groups	Collagen content (µg/mg tissue)	
	Day 3	Day 7
untreated	42.65 ± 4.08	48.72 ± 0.98
gel base	40.52 ± 1.99	49.35 ± 4.99
0.05% ECa 233	46.26 ± 3.61	53.63 ± 3.29
0.1% Eca 233	48.23 ± 3.61	55.21 ± 3.19
0.5% Eca 233	50.77 ± 4.22	58.07 ± 0.88

Table 6 : The effect of different concentration of ECa 233 on collagen content in diabetic rats. The values are expressed as the mean ± S.E.M. (n=6). Statistical analyses were performed by one-way ANOVA and Duncan for comparison. A significant value of p less than 0.05 was considered as statistically significant.

* Significantly different from value in untreated group.

Significantly different from value in gel base group.

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Chulalongkorn University Animal Care and Use Committee

Certificate of Project Approval	☐ Original ☐ Renew
Animal Use Protocol No. 09-33-004	**Approval No. 09-33-004**
Protocol Title Effect of Eca 233, standard extract of *Centella asiatica*, on incision wound healing in non-diabetic and diabetic rats	
Principal Investigator Mayuree Tantisira, Ph.D.	
Certification of Institutional Animal Care and Use Committee (IACUC) This project has been reviewed and approved by the IACUC in accordance with university regulations and policies governing the care and use of laboratory animals. The review has followed guidelines documented in Ethical Principles and Guidelines for the Use of Animals for Scientific Purposes edited by the National Research Council of Thailand.	
Date of Approval January 30, 2009	**Date of Expiration** January 30, 2010
Applicant Faculty/Institution **Faculty of Pharmaceutical Sciences, Chulalongkorn University, Phyathai Rd., Pathumwan BKK-THAILAND. 10330**	
Signature of Chairperson	**Signature of Authorized Official**
Name and Title **WITHAYA JANTHASOOT** **Chairman**	**Name and Title** **RUNGPETCH SAKULBUMRUNGSIL, Ph.D.** **Associate Dean (Research and Academic Service)**
The official signing above certifies that the information provided on this form is correct. The institution assumes that investigators will take responsibility, and follow university regulations and policies for the care and use of animals. *This approval is subjected to assurance given in the animal use protocol and may be required for future investigations and reviews.*	

BIOGRAPHY

Miss Hataichanok Tanintaraard was born on December 22, 1980 in Sakonnakhon province, Thailand. She had graduated with the Bachelor Degree of Nursing Science from the Faculty of Nursing, Khonkaen University in 2002 and then worked at Bumrungrad International Hospital, Bangkok for five years. In 2007, she became a graduate student in the master's degree of science program in Pharmacology, Interdisciplinary Program, Graduate School, Chulalongkorn University.

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