ANALYSIS OF HARUAN (C*hanna striatus*) WATER EXTRACT AND THE STUDY OF HARUAN AEROSOL ON WOUND HEALING

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ANALYSIS OF HARUAN (C*hanna striatus*) WATER EXTRACT AND THE STUDY OF HARUAN AEROSOL ON WOUND HEALING

By

LIA LAILA

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In the name of Allah, the Most Gracious, Most Merciful.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
AAbA	Alpha aminobutyric acid
ADP	Adenosine diphosphate
ANOVA	Analysis of Variance
AOAC	Association of Official Analytical Chemists
A.S.T.M.	American Society for Testing and Materials
ATCC	American Type Culture Collection
ATP	Adenosine triphosphate
AQC	6-aminoquinolyl-N-hydroxysuccinimidyl carbamate
BC	Before Century
BCS	Bovine Calf Serum
bFGF	Basic Fibroblast Growth Factor
^{o}C	Degree of Centigrade
cm	Centimeter
cm^2	Square centimetre
CV	Coefficient of variance
DEJ	Dermal-epidermal junction
DHA	Docosahexaenoic acid
DMEM	Dulbecco's Modified Eagle's medium
ECM	Extracellular matrix
EGF	Epidermal growth factor
EMEM	Eagle's Modification Essential Medium
EPA	Eicosapentaenoic Acid

Eq.	Equation
FAME	Fatty acid methyl ester
FBS	Fetal Bovine Serum
FID	Flame Ionization Detector
g	Gram
GC	Gas chromatography
h	Hour
HPLC	High performance liquid chromatography
HPMC	Hydroxypropylmethylcellulose
HWE	Haruan water extract
ICH	International Conference of Harmonization
IFN	Interferon
IGF	Insulin-like growth factor
IL	Interleukin
KGF	Keratinocyte growth factor
kPa	Kilo Pascal
L	Liter
LOD	Limit of detection
LOQ	Limit of quantification
LVI	Limited volume insert
MCP	Monocytes chemoattractant protein
min	Minute
MMP	Matrix metalloproteinase
µg	Microgram
µL	Microliter

µm	Micrometer
mL	Milliliter
MUFA	Monounsaturated fatty acid
N	Number of replications
ng	Nanogram
NO	Nitric oxide
PDGF	Platelet derived growth factor
PEG	Polyethylenglycol
PII	Primary Irritation Index
pmol	Picomole
PUFA	Polyunsaturated fatty acid
R^2	Correlation coefficient
RSD	Relative standard deviation
s	Second
SD	Standard deviation, Sprague-Dawley
SFA	Saturated fatty acid
SPARC	Secreted protein acidic and rich in cysteine
TGF	Transforming growth factor
TIMP	Tissue inhibitor of metallo-protease
TNF	Tumor necrosis factor
USP	United State Pharmacopoeia
VEGF	Vascular endothelial growth factor

LIST OF PUBLICATIONS

Journal Publication:

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1. Lia Laila, Saringat Bin Bai @ Baie, Azmin bin Mohd. Noor, Salizawati binti Muhamad Salhimi ,Omeed M. Hassan, Febriyenti, 2009, Biological Reactivity Test Of Haruan (*Channa striatus*) Spray As Wound Dressing, 2nd Colaborative Conference UNAIR-USM 2009, Surabaya- Indonesia, page 288.

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3. Lia Laila, Febriyenti, Salizawati M.Salhimi, Saringat Baie. 2011. Fatty Acid Composition Of Haruan (*Channa striatus*) Aqueous Extract For Wound Healing. 1st National Postgraduate Conference in Molecular Medicine, Renaissance Hotel, Kota Bharu-Kelantan, 13-14 April 2011.

✓ Oral Presentation

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2. Lia Laila, Salizawati Muhamad Salhimi, Saringat Baie. 2010. Effect Of Haruan Aqueous Extracts in Wound Closure Of NIH/3T3, HFF-1 and JB6 Cl 30-7B Cell Lines. International Conference on Natural Products, Bayview Beach Resort, Penang, 11-12 December 2010 published in Malaysian Journal of Pharmaceutical Sciences Suppl. 1 (2010) page 48.

Analisis Ekstrak Air Ikan Haruan (*Channa striatus*) dan Kajian Haruan Aerosol terhadap Penyembuhan Luka

ABSTRAK

Ciri mempercepat penyembuhan luka ekstrak air ikan haruan telah dikaji. Kajian *in vitro* dan *in vivo* mengunakan titisan sel dan model haiwan telah dipilih untuk mencerminkan kesan penyembuhan. Analisa ekstrak air ikan haruan telah dijalankan untuk menentukan kandungan asid amino dan asid lemak. Penentuan asid amino dalam ekstrak air ikan haruan telah dijalankan menggunakan kaedah hidrolisis asid, diikuti oleh derivatisasi AQC pra-turus dan dianalisis menggunakan kromatografi cecair prestasi tinggi yang dilengkapi dengan pengesan pendarfluor, sementara penentuan asid lemak dilakukan menggunakan kaedah metilasi asid dengan kromatografi gas yang dilengkapi dengan pengesan pengionan nyalaan . Pengesahan kaedah juga telah dibangunkan untuk memastikan data yang diperolehi boleh dipercayai dan sah bagi kedua-dua analisis asid amino dan asid lemak. Keputusan parameter pengesahan menunjukkan bahawa semua data yang diperolehi berada dalam julat mengikut nilai yang boleh diterima oleh garis panduan ICH. Penentuan asid amino menunjukkan bahawa ekstrak air ikan haruan mengandungi sekurang-kurangnya 16 asid amino yang mana glisin dan asid glutamik (masing-masing 17,51% dan 14,32% daripada jumlah asid amino) adalah asid amino utama dalam ekstrak. Sebaliknya, tirosin diikuti oleh histidin adalah asid amino yang paling sedikit. Analisis asid lemak menunjukkan bahawa terdapat sekurang-kurangnya 18 asid lemak yang dikenal pasti dalam ekstrak air ikan haruan yang dikategorikan sebagai asid lemak tepu, asid lemak mono tak tepu, dan asid lemak poli tak tepu. Asid lemak yang paling banyak di dalam ekstrak air ikan haruan asid adalah

asid palmitik diikuti oleh asid palmitoleik (masing-masing 39,12% dan 11,28% daripada jumlah asid lemak). Sementara itu, asid laurik dan asid erusik merupakan asid lemak yang paling sedikit. Kajian *in vitro* telah dijalankan menggunakan kaedah penyembuhan luka gores untuk menilai kesan ekstrak air ikan haruan dalam penghijrahan sel-sel yang cedera. Hasilnya menunjukkan bahawa ekstrak air ikan haruan menggalakkan penutupan luka sel epidermis tikus dengan kadar 5% berbanding dengan kawalan. Kadar ini kemudiannya dipilih untuk diformulasikan dalam bentuk dos aerosol dan digunakan untuk kajian *in vivo*. Dalam kajian ini ujian kereaktifan biologi dan kajian keberkesanan penyembuhan luka dijalankan. Ujian kereaktifan biologi telah dijalankan ke atas model haiwan mencakupi tiga jenis eksperimen yaitu ujian Kerengsaan Kulit Rendah, ujian Intracutaneous, dan ujian Suntikan Sistemik. Hasil kajian menunjukkan bahawa aerosol Haruan tidak memberi sebarang tindak balas yang signifikan kepada semua ujian. Penyiasatan kesan aerosol Haruan sebagai penutup luka dalam proses pemulihan dilakukan menggunakan tikus Sprague-Dawley di mana luka hirisan dan luka bakar telah dibuat ke atas belakang haiwan-haiwan. Aerosol Haruan diuji dan dibandingkan dengan formula kosong sebagai kawalan. Ujian kekuatan tegangan, pengecutan luka dan parameter histopathologi telah dipilih sebagai hasil untuk mengukur kejayaan penyembuhan. Hasil kajian menunjukkan bahawa aerosol Haruan memberi penguncupan luka yang signifikan pada minggu pertama penyembuhan dan meningkatkan proses pemulihan yang disokong oleh data histopathologi. Dari semua keputusan, dapat disimpulkan bahawa aerosol haruan dengan ekstrak air ikan haruan sebagai bahan aktif mempunyai potensi yang besar untuk menggalakkan proses penyembuhan luka dan mempercepatkan masa penyembuhan dengan prosedur terapeutik yang selamat dan mudah.

Analysis of Haruan (*Channa striatus*) Water Extract and the Study of Haruan Aerosol on Wound Healing

ABSTRACT

The enhancement of wound healing properties of haruan water extract has been studied. The *in vitro* and *in vivo* studies using cell line and animal model were carried out to evaluate the effectiveness of the extract. Analytical study of haruan water extract was conducted to determine the amino acid and fatty acid contents. The determination of the amino acid in haruan water extract was carried out using acid hydrolysis method, followed by AQC pre-column derivatization and analysed using high performance liquid chromatography equipped with fluorescence detector, while the fatty acid determination was performed using acid methylation method with gas chromatography equipped with flame ionization detector. A method validation was also established to ensure the data obtained reliable and valid for both amino acid and fatty acid analysis. The results of validation parameters showed that all the data obtained were in the range of acceptable value accordance to ICH guideline. The results showed that haruan water extract containing at least 16 amino acids of which glycine and glutamic acid were the major component of the amino acids (17.51% and 14.32% respectively of total amino acid) in the extract. While, tyrosine followed by histidine was the least amino acids. The fatty acid analysis showed that there were at least 18 fatty acids identified in haruan water extract which were categorized as saturated fatty acid, monounsaturated fatty acid, and polyunsaturated fatty acid. The most abundant fatty acid in haruan water extract is palmitic acid followed by palmitoleic acid (39.12% and 11.28% respectively of total fatty acid). While lauric acid and erucic acid is the least fatty acid found in the

extract. The *in vitro* study was carried out using wound healing scratch assay to evaluate the effect of haruan water extract in the migration of wounded cells. It showed that haruan water extract promoted the wound closure of mouse epidermal cells at the concentration of 5% as compared to its control. This concentration was then selected for the formulation of aerosol dosage form and was used in the *in vivo* study. In this study a biological reactivity test and wound healing efficacy study were carried out. Biological reactivity test was carried out on animal models encompass three types of experiments which are the Primary Skin Irritation test, the Intracutaneous test, and the Systemic Injection test. The results showed that Haruan aerosol gave no significant response to all the tests. The investigation of the effect of Haruan aerosol as wound dressing in the healing process was performed in Sprague-Dawley rats where full-thickness incision wound and burn wound were made on the back of the animals. Haruan spray was tested and compared with blank formula as control. Tensile strength test, wound contraction and histopathology parameters were selected as the outcomes to measure the success of the healing. The results showed that haruan aerosol gave a highly significant wound contraction in the first week of healing and improved the healing process which was supported by the histopathology data. From all of the results, it can be concluded that Haruan aerosol with haruan water extract as active ingredient has a great potential to promote wound healing process and accelerate the healing time with safe and simple therapeutic procedure.

CHAPTER 1
INTRODUCTION

1.1. HARUAN FISH

Haruan fish is a well known fresh water fish in Malaysia. It is a species of snakehead that scientifically known as *Channa striatus*. It is also known as stripped snakehead, common snakehead, and snakehead murrel. This fish belongs to the family of Channidae and it is mostly distributed in many tropical countries such as Malaysia, Indonesia, Singapore, Thailand, Indochina and India (Lee and Ng, 1994, Lowe-McConnell, 1987, Alfred, 1966, Qasim and Bhatt, 1966, Fowler, 1938, Maxwell, 1921).

Traditionally, haruan fish is used by rural folks especially mothers after giving birth and people who have undergone surgery. They believe that by eating this fish, they will gain strength and promote wound healing. Haruan is also encouraged to be taken by Chinese women after giving birth as food supplement during confinement (Koon and Karim, 2005).

In the past 15 years, many studies have been conducted in relation to haruan fish. The results showed that haruan fish has many other advantages not only in wound healing. Several findings on studies of haruan fish are tabulated in Table 1.1.

Table 1.1: Role of haruan in several studies

Pharmacology Activity	Part that used	Result	Reference
Wound healing	Fish extract Mucus and Roe	Positive Positive	Baie and Sheikh (2000a, 2000b) Mat Jais *et al.*(1998)
Antinociceptive	Fillet extract and mucus extract Aqueous supernatant extract	Positive	Mat Jais *et al.* (1997), Dambisya *et al.* (1999) Zakaria *et al.* (2007)
Anti-inflammatory for osteoarthritis	Fillet extract	Positive	Michelle *et al.* (2004)
Lowering blood glucose and cholesterol level	Fillet extract	Negative	Mat Jais *et al.* (2002)
Mild antibacterial and antifungal	Fish extract	Positive	Mat Jais (2007)
Platelet aggregation and blood clotting	Fish extract	Positive	Unpublished work by Mat Jais

1.2. SKIN

Skin is the largest and most complex organ that covers the whole body surface of a human body (15% of total adult body weight). It has multiple vital functions i.e. protection against external physical, chemical, and biological aggressions; maintenance of its homeostatis; storage of nutrients; excretion of substances; the synthesis of vitamin; and sensing (Martini, 2007, Kanitakis, 2002, Walters and Roberts, 2002). It consists of 3 layers including from top to bottom namely epidermis, dermis and hypodermis which organise several tissues of various cells (epithelial, connective, muscular and nervous) (Kanitakis, 2002).

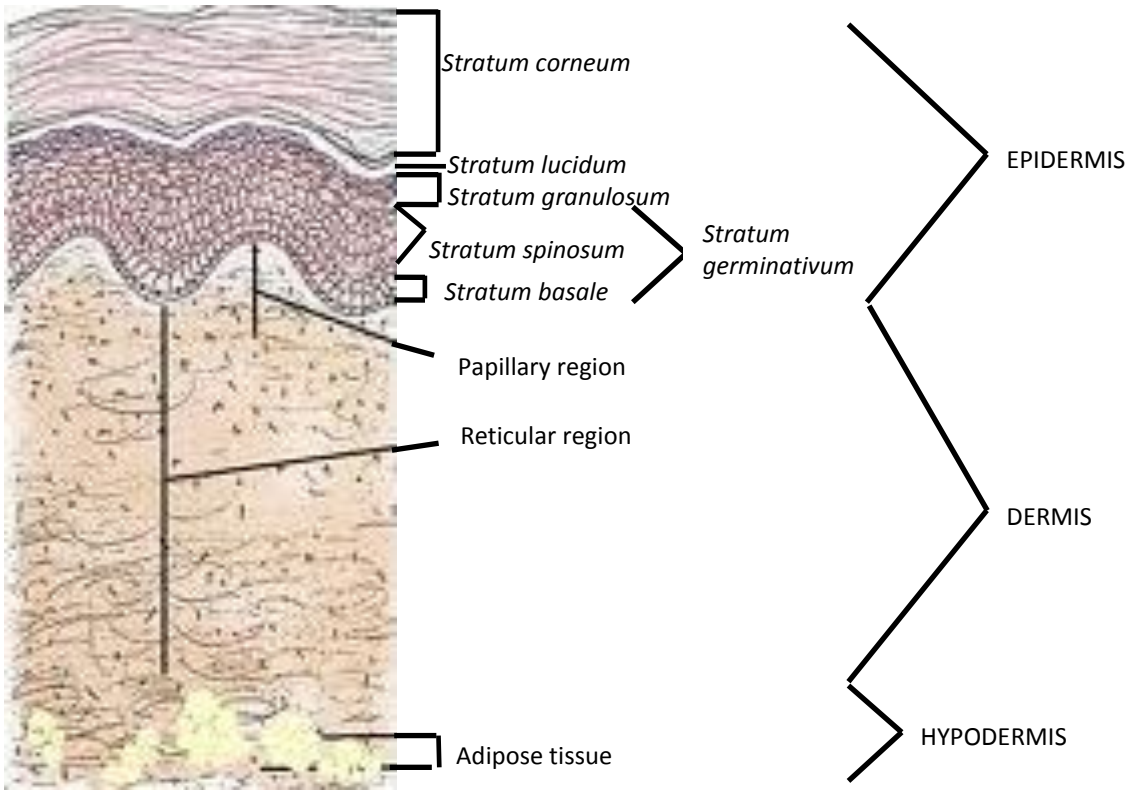

Figure 1.1: Anatomic structure of normal skin (adopted from www.google.com)

1.2.1. Epidermis

Epidermis is made up of several cells types, the majority (90 to 95%) is keratinocyte cells, the rest (5 to 10%) are Langerhans cells, melanocytes and Merkel cells (Kanitakis, 2002). Epidermis has five layers or zones that are recognizable that is *stratum corneum* (nonviable epidermis), *stratum lucidum*, *stratum granulosum*, and *stratum germinativum* which is divided into *stratum spinosum* and *stratum basale* (cylindricum) (Di-Fiore, 1967). The average thickness of epidermis is approximately 100 µm (Kanitakis, 2002). In the epidermis layers, *stratum corneum* has an important role. It is the very outside layer of the skin. The thickness of *stratum corneum* is approximately 10 to 20 µm. The functions of the epidermis are based on the layers

that organise it. *Stratum corneum* as the nonviable epidermis is a barrier to a physical interaction as well as acts to prevent the loss of the internal body components, particularly water, to the external environment. The functions of viable epidermis are to form and maintain the generation of *stratum corneum*, metabolism of substrates, including foreign substances, synthesis of melanin for skin pigmentation and as protection against the sun (Walters and Roberts, 2002).

1.2.2. Dermis

The second layer is the dermis, a supportive, compressible and elastic connective tissue which protects the epidermis (Kanitakis, 2002). The main cells present in the dermis are fibroblast cells which make up 70% of dermis as a collagenous fibre tissue for stretch and for strength, as well as abundance in blood vessels and specialized nerve endings (Sherwood, 2008, Kanitakis, 2002). The thickness of dermis is about 0.1 to 0.5 cm, varying depending on the anatomic location. The functions of dermis are to provide the nutritive, immune, support system for the epidermis, and play a role in the temperature, pressure, and pain regulation. In the dermis, there are extensive vascular network which provide the skin with nutrition, repair wounds and immune response. The lymphatic system regulates pressure, mobilises defence mechanism and waste removal. There are also nerve fibres that give the skin with sensory of pressure, pain and temperature (Walters and Roberts, 2002).

1.2.3. Subcutaneous layer (hypodermis)

Subcutaneous layer is the deepest part of the skin consisting of fat cells (adipocytes), fibroblast and macrophages. The functions of subcutaneous tissue are heat insulator, shock absorber, energy storage, anchoring the skin to the underlying muscle; carrying the vascular and neural system to the skin (Kanitakis, 2002; Walters and Roberts, 2002).

1.2.4. Dermal – Epidermal Junction (DEJ)

This junction is a complex basement membrane synthesised by the basal epithelial cells and dermal fibroblasts. This junction is important for supporting the epidermis to the dermis and regulating the exchange of metabolic products between the two layers. In wound healing process, this junction plays an important role in serving as a support for the migration of epithelial cells (Kanitakis, 2002).

1.3. WOUND DEFINITION

There are many definitions for wound. Enoch and Leaper (2007) defined wound as *a break in the epithelial integrity of the skin and may be accompanied by disruption of the structure and function of the underlying normal tissue.* Winkler and Makowski (2005) defined wound as *any physical break in tissue continuity*. Cockbill (2002) defined wound as *any process which leads to the disruption of the normal architecture of a tissue*. Wound Healing Society defined wound as *a disruption of normal anatomic structure and function (*Lazarus *et al.*, 1994). Stephen Thomas

(1990) defined wound *as a defect or break in the skin that results from physical, mechanical or thermal damage, or that develops as a result of the presence of an underlying medical or physiological disorder.*

As a conclusion, it can be summarised that a wound is a disruption or a break in the skin or tissue that affects the normal anatomic structure and function resulting from physical, mechanical or thermal damage.

1.4. TYPE OF SKIN WOUND

According to the number of skin layers affected, wound can be classified into 3 parts (Cockbill, 2002):

a. Superficial wound

   The damage caused is limited to the epithelial tissue alone or epidermis layer and the healing will go rapidly by regeneration of epithelial cells.

b. Partial thickness wound

   The disruption is involved in the deeper dermal layer and included vessel damage.

c. Full thickness wound

   This type of wound affects the subcutaneous layer and beyond. It usually takes longer time to heal.

The common classes of wound according to Thomas (1990) are:

1.4.1. Mechanical injuries

The types of wound that included in this class are:

a. Abrasions (grazes)

   The wound is generally caused by friction between skin and second harder or rougher surface.

b. Lacerations (tears)

   It is more severe than abrasions which involve the underlying tissues.

c. Penetrating wound

   This type of wound is generally caused by any sharp or pointed object which penetrated the skin beneath such as caused by knives, bullets or other sharp objects.

d. Bites

   It is caused by animals or humans.

e. Surgical wound

   This type represents mechanical injury caused by surgery process.

1.4.2. Burns and chemical injuries

There are several types of burn injury through aetiology: thermal, chemical, electrical, and radiation. The most common injury is caused by thermal. The classification of burn wound type depends on how deep the skin layer is affected. They are usually termed as degree of the burn.

a. First degree: burns involve only the epidermis and superficial layers of the dermis.

b. Second degree: most surface of the epithelium is destroyed with much of the deeper dermal layer affected.

c. Third degree: all elements of the skin are destroyed.

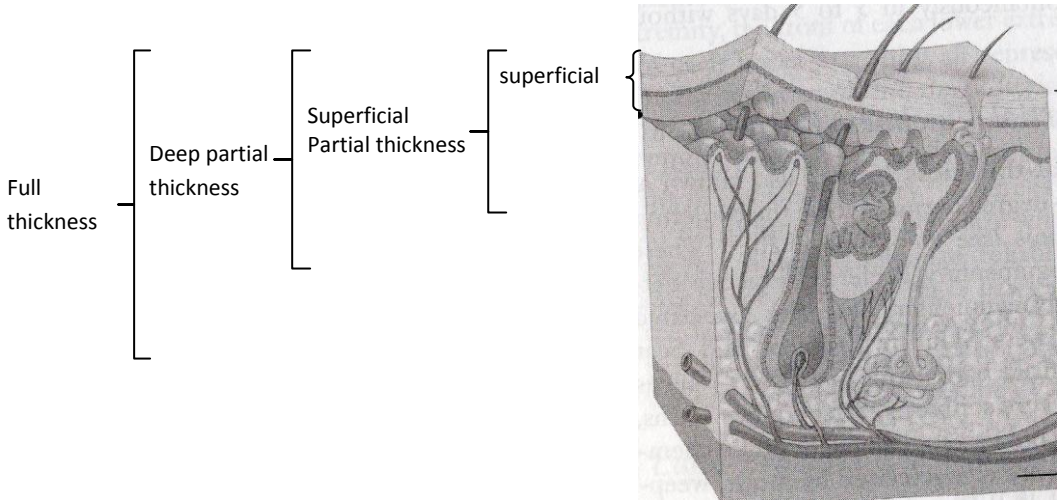

Figure 1.2: Depth of tissue in burn wound injuries (adopted from Myers, 2008)

1.5. WOUND HEALING

Wound healing is a complex, dynamic, interactive and collaborative process involving cellular and biochemical processes like variety of soluble mediators, cells, extracellular matrix that relate to achieve a common goal of tissue regeneration in the restoration of anatomic and function of injured part (Boateng *et al.*, 2008, Kumar *et al.*, 2004, Steed, 2003, Singer *et al.*, 2000, Lazarus *et al.*, 1994).

There are several types of wound healing based on the intentions:

- Primary healing (healing by first intention)

  Most of clean surgical incision or laceration wounds with minimal bacterial contamination and no significant tissue lost are healed by primary closure (Enoch and Leaper, 2007, Halloran and Slavin, 2002, Thomas, 1990). The edges of the wound are directly closed with sutures, tissue glue, tapes or mechanical devices within 12-24 hours (Enoch and Leaper, 2007).

  This primary healing is initiated by the movement of epithelial cells from the edges of the wound to move closer to each other (Cockbill, 2002). The epithelial cells are proliferated in dermal – epidermal junction (Halloran and Slavin, 2002).

- Delayed primary healing

  This type of healing is similar to the primary healing. The only difference is that there is contamination of bacteria or the wound closed a few days after the injury which delayed the healing process for several days (Enoch and Leaper, 2007).

- Secondary healing (healing by second intention)

  This healing type is also known as open granulation (Thomas, 1990). The tissue damages are often greater (Halloran and Slavin, 2002). The skin wound loss of soft tissue extensively as a result of trauma, surgery or severe burn (Enoch and Leaper, 2007, Cockbill, 2002, Thomas, 1990). The healing begins

with the creation of granulation tissue at the base of the wound (Cockbill, 2002).

- Healing of superficial wounds

  The injury involves only the epithelium and the superficial of dermis such as superficial burn, split-thickness donor graft sites, and abrasions. Thus the healing occurs only by epithelialisation (Enoch and Leaper, 2007).

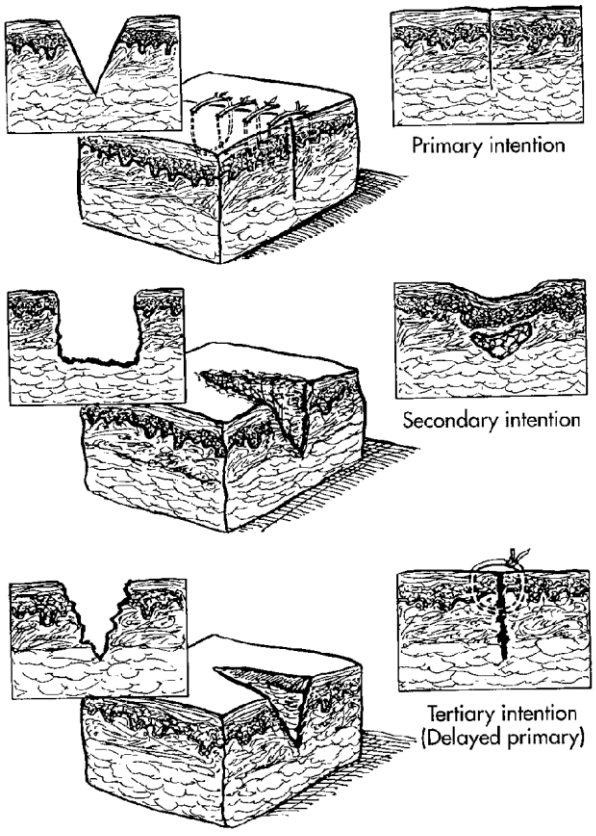

Figure 1.3: Types of wound healing intention (adopted from Strodtbeck, 2001)

Wound healing process starts immediately after injury has occurred (Cockbill, 2002). The healing process is an overlapping phase which is also a continual process that

conceptually moving toward to the next phases (Lorenz and Longaker, 2008). Some researchers divide the healing process into three phases (Kondo and Ishida, 2010, Lorenz and Longaker, 2008, Grazul-Bilska *et al.*, 2003, Steed, 2003, Cockbill, 2002), four phases (Guo and DiPietro, 2010, Menke *et al.*, 2008, Eccleston, 2007, Enoch and Leaper, 2007, Oberyszyn, 2007, Gosain and DiPietro, 2004, Williamson and Harding, 2004), and five phases (Boateng *et al.*, 2008, Monaco and Lawrence, 2003, Prathiba and Gupta, 2000).

1.5.1. Haemostasis phase

Usually immediately after skin injury, bleeding will occur to flush out bacteria and antigen from the wound (Boateng *et al.*, 2008). This bleeding process will be stopped by haemostasis. When a wound appears, blood quickly escapes from the blood vessels. Thus, vascular response occurs immediately after the skin gets injured. The first response is vasoconstriction of blood vessel to reduce the flow of blood (Monaco and Lawrence, 2003, Irvin, 1985). Vasoconstriction occurred by the liberation of adrenaline to the peripheral circulation when the dermis is penetrated (Monaco and Lawrence, 2003).

Bleeding is stopped with the clot formation produced by platelets aggregation. Platelets aggregation is activated by contacting with thrombin and mature collagen (Thomas, 1990). During the aggregation and adhesion, platelets release some content of alpha granules (contain variety of immunomodulatory and proteinaceous factor include albumin, fibrinogen, fibronectin, IgG, coagulation factors V and VIII, and growth factors), dense bodies (include calcium, serotonin, ADP and ATP), and

lysosomal enzymes that draw more platelets to the injured area (Monaco and Lawrence, 2003).

At the same time, thromboplastin liberated from the wounded cells will cleave fibrinogen to fibrin monomers to produce fibrin network. Platelets aggregation and fibrin network together form a strong clot that gives strength and support to the injured wound (Keast and Orsted, 1998, Thomas, 1990).

1.5.2. Inflammatory phase

The inflammatory phase occurs about the same time with haemostasis, thus in several references, haemostasis and inflammation both appear in the first phase (Boateng *et al.*, 2008, Lorenz and Longaker, 2008, Cockbill, 2002, Singer and Clark, 1999, Witte and Barbul, 1997). Sometimes starts within a few minutes after injury and lasts for 3 to 4 days (Boateng *et al.*, 2008, Lorenz and Longaker, 2008, Keast and Orsted, 1998).

The symptoms of inflammation process include redness, swelling, heat and pain occurring at the wound site (Diegelmann and Evans, 2004, Monaco and Lawrence, 2003, Cockbill, 2002, Keast and Orsted, 1998). These signs start to occur when vasodilatation appeared and gaps occurred between the vascular endothelial cells, which permit the plasma to fill up the site of injury via diapedesis and causing the area around the wound to become swollen (known as edema) (Cockbill, 2002). The increase in surface area of the dilated vessel results in greater visualibility of the capillaries making the injured wound reddish in colour, while the heat transfer to the

surrounding tissues making the wound area warm to touch. The agents that are responsible for the vasodilatation and vascular permeability action are leukotriene, serotonin, bradykinin, prostaglandin and in particular histamine (Lorenz and Longaker, 2008, Grazul-Bilska *et al.*, 2003, Monaco and Lawrence, 2003, Thomas, 1990, Hunt, 1988, Peacock, 1984).

The goal of the inflammatory phase is to clean up all the debris that occurred at the wound area. Neutrophils start to appear in the wound site within hours followed by macrophages (Thomas, 1990). Both of these agents have roles in debris removal and bacteria ingestion through phagocytosis and intracellular killing (Martin *et al.*, 1992). At the late inflammatory phase (2 to 3 days), the macrophages dominate the wound site replacing the neutrophils and release cytokines (include growth factors) also immunomodulatory factors that initiate the next stage of healing process (Lorenz and Longaker, 2008, Monaco and Lawrence, 2003).

1.5.3. Proliferation phase

When the inflammation process is near to the end, the proliferation phase begins on day 3 and lasts until 2-3 weeks (approximately 21 days) after injury (Enoch and Leaper, 2007, Keast and Orsted, 1998, Hanna and Giacopelli, 1997), depending on the site and size of the wound (Peacock, 1984). This phase is characterized by three parts: migration of fibroblast cells, deposition of extracellular matrix (ECM) and formation of granulation tissue. Epithelialisation process will appear at the end as a representative of final stage of the proliferation phase (Enoch and Leaper, 2007).

a. Fibroblast Migration

Fibroblasts are the key factors in the proliferation phase. Fibroblasts migrate to the wound site around 2- 4 days after injury (Enoch and Leaper, 2007). Proliferation and migration of fibroblast cells are induced by growth factors and cytokines (Lorenz and Longaker, 2008, Diegelmann and Evans, 2004, Kumar *et al.*, 2004, Grazul-Bilska *et al.*, 2003, Monaco and Lawrence, 2003, Singer and Clark, 1999, Walsh, 1998, Lawrence and Diegelmann, 1994). Fibroblasts move into the wound using the fibrin and fibronectin matrix. Once fibroblast cells are divided and proliferated, they start the synthesis and secretion of extracellular matrix components (Lorenz and Longaker, 2008).

b. Extracellular Matrix (ECM) Deposition

The deposition of ECM in the wound area is to provide a substratum for cell adhesion and also for the regulation of growth, movement, and differentiation of the cells within it (Enoch and Leaper, 2007). The major cells related to ECM are the fibroblasts which are responsible for the production of ECM components (Lorenz and Longaker, 2008, Cockbill, 2002). The components in ECM are described as follow:

1. Fibrous structural proteins

The structural proteins in ECM are collagen and elastin (Enoch and Leaper, 2007, Cockbill, 2002). Collagen and elastin are synthesised by the fibroblast (Boateng *et al.*, 2008, Enoch and Leaper, 2007, Cockbill, 2002, Hanna and Giacoppelli, 1997). Collagen molecules also known as tropocollagen is consisted of three polypeptides

α- chain woven into a rope-like triple helix. Each α-chain has a repetitive sequence of amino acids where every third amino acid is glycine (Enoch and Leaper, 2007; Halloran and Slavin, 2002). Collagens are differentiated to 11 types based on the difference of α-chain (Halloran and Slavin, 2002). Only collagen I-III gives the fibrillar form (Enoch and Leaper, 2007, Halloran and Slavin, 2002, Brinckmann *et al.*, 1994) and creates most of the connective tissue in the healing wounds. The other collagen types (e.g. type IV) are non-fibrillar and contribute as the component of basement membrane (Enoch and Leaper, 2007, Halloran and Slavin, 2002). Elastin is a highly insoluble protein that gives skin the elasticity (Monaco and Lawrence, 2003, Eyre *et al.*, 1984).

2. Adhesive Glycoprotein

Adhesive glycoproteins are molecules that bind to the cells via a cell surface receptor called integrin and also other extracellular matrix components (Lorenz and Longaker, 2008, Enoch and Leaper, 2007). Glycoprotein has two binding sites, one for collagen and another for cell surfaces and it influences attachment, spreading and migration of cells (Enoch and Leaper, 2007, Irvin, 1985). These molecules include fibronectin, laminin and vitronectin and are known as attachment factors (Davidson, 2002).

3. Glycosaminoglycans and Proteoglycans

Glycosaminoglycans are polysaccharide chains which include hyaluroran or hyaluronic acid, dermatan sulphate, chondroitin sulphate, heparin sulphate, and keratin sulphate. Hyaluroran promotes cell migration in early granulation tissue (Toole, 1991).

Proteoglycan contains a core protein, one of which is glycosaminoglycan chain (e.g. chondroitin -4 sulphate and dermatan sulphate) and is bound covalently (Clark, 1996). Proteoglycans can alter the cell growth and cell differentiation (Enoch and Leaper, 2007). Proteoglycan that contained chondroitin-4-sulphate and dermatan sulphate increase in the second week of healing process (Bentley, 1967), and this proteoglycan is produced by mature fibroblast (Bronson *et al.*, 1988).

4. Matricellular proteins

Matricelluar proteins are a group of proteins including galectins, osteopontin, secreted protein acidic and rich in cysteine (SPARC), tenascins, thrombospondins, and vitronectin (Midwood *et al.*, 2004, Davidson, 2002). Most of these proteins interact via integrin and response to certain growth factors which also affect the cell migration, contraction of matrix and angiogenesis (Midwood *et al.*, 2004).

c. Granulation Tissue Formation

Granulation tissue is formed on day 3 – 5 (around day 4) after injury. Granulation tissue consists of macrophages, proliferating fibroblast and new capillaries in a loose ECM (Enoch and Leaper, 2007, Cockbill, 2002, Singer and Clark, 1999). Granulation tissue has a pink or rossy, soft, granular gross appearance (Myers, 2008, Enoch and Leaper, 2007) which is created by the formation of new blood vessel and extracellular matrix.

The new formation of blood vessel from pre-existing vessels is called angiogenesis (Bicknell and Harris, 2004, Augustin, 2001) or neovascularisation (Enoch and Leaper, 2007). Angiogenesis is stimulated by the vascular endothelial growth factor

(VEGF) and basic fibroblast growth factor (bFGF) (Bicknell and Harris, 2004), which are mainly produced by macrophages (Kondo and Ishida, 2010). It is important to maintain the newly formed granulation tissue because it provides oxygen and nutrition for the fibroblast to proliferate and to produce collagen (Tonnesen *et al.*, 2000, Singer and Clark, 1999).

d. Epithelialisation

A single layer of epidermal cells migration occurs in the few hours after injury from the edge of the wound (uninjured area) covering the bare area which known as epiboly process (Lorenz and Longaker, 2008, Enoch and Leaper, 2007). Approximately 12 hours after the initiation of migration, there is a sign of increase in mitotic activity within epithelial cells of the wound edge. Epithelialisation process is altered by several growth factors like keratinocyte growth factor, epidermal growth factor and basic fibroblast growth factor (Enoch and Leaper, 2007; Monaco and Lawrence, 2003). Epithelial cells will continue to proliferate, migrate and differentiate to make a protective outer layer or stratum corneum which allows the restoration of surface integrity (Enoch and Leaper, 2007, Grazul-Bliska, *et al.*, 2003, Keast and Orsted, 1998).

1.5.4. Maturation or Remodelling phase

This stage marks the end of the healing process since all of the aspects included in the healing process is back to near its pre-wounding level (Braiman-Wiksman *et al.*, 2007). In this stage, wound contraction occurs to close the gap of the wound. The

contraction of wound had been investigated by Gabbiani *et al.* (1971) and they found that the contraction was initiated by the modified fibroblast which is known as the myofibroblast.

Wound remodelling starts approximately 21 days after injury (Enoch and Leaper, 2007; Monaco and Lawrence, 2003). Collagen synthesis begins to deprive which regulated by γ-interferon, tumor necrosis factor (TNF-α) and collagen matrix itself (Monaco and Lawrence, 2003). The matured collagens change its structure during the alteration from granulation tissue to scar to perform more stable state (Singer and Clark, 1999). This collagen remodelling is also affected by collagenolysis process which is also important in the removal of collagen debris in injured tissue (Halloran and Slavin, 2002). Collagenolysis happened in a lower rate with the help of an enzyme called collagenase which is a member of matrix metalloproteinases (MMPs) family. MMPs family is produced by fibroblast, endothelial cells, neutrophils and macrophages (Diegelmann and Evans, 2004; Williamson and Harding, 2004; Halloran and Slavin, 2002; Singer and Clark, 1999). Other enzymes of MMPs members are gelatinases and stromelysins which are also involved in matrix degradation (Grazul-Bliska, *et al.*, 2003).

Although this is the last phase, it can last from 3 weeks up to two years (Lorenz and Longaker, 2008, Eccleston, 2007, Cockbill, 2002, Keast and Orsted, 1998) or even several years (Guo and DiPietro, 2009, Lawrence and Diegelmann, 1994). At this phase, wounded skin gets back its strength and elasticity (Braiman-Wiksman *et al.*, 2007). However, the strength of the scar tissue will only regain 70% to 80% of the original (unwounded) skin (Enoch and Leaper, 2007, Diegelmann and Evans, 2004, Williamson and Harding, 2004, Cockbill, 2002, Levenson *et al.*, 1965).

1.6. GROWTH FACTORS AND CYTOKINES

The cells that are involved in the wound healing process include fibroblasts, epithelial cells and vascular endothelial cells which secrete various growth factors and cytokines (Walsh, 1998). Growth factor is made up of amino acids which then form polypeptide (Martin *et al.*, 1992). There are several growth factors and cytokines that play a significant role in the wound healing process. The contribution of these growth factors and cytokines in wound healing can be seen in Table 1.2 and Table 1.3.

Table 1.2: Growth Factors in Wound Healing

Growth Factors	Sources	Functions	References
Transforming Growth Factors (TGF) β_1 and β_2	Macrophages, platelets, fibroblasts, keratinocytes, bone cells	Chemoattractant to fibroblast, monocytes, lymphocytes	Lorenz and Longaker (2008), Diegelmann and Evans (2004), Kumar *et al.* (2004), Grazul-Bliska, *et al.* (2003), Monaco and Lawrence (2003), Singer and Clark (1999), Walsh (1998), Lawrence and Diegelmann (1994)
		Proliferation of epithelial cells, macrophages, lymphocytes, fibroblast	Lorenz and Longaker (2008), Diegelmann and Evans (2004), Kumar *et al.* (2004), Grazul-Bliska, *et al.* (2003), Lawrence and Diegelmann (1994)
		Stimulates keratinocytes migration	Kumar *et al.* (2004), Singer and Clark (1999)
		Induce synthesis of extracellular matrix proteins	Lorenz and Longaker (2008), Diegelmann and Evans (2004), Singer and Clark (1999), Walsh (1998)
		Induce fibroblast to secrete tissue inhibitor of metallo-protease (TIMP)	Diegelmann and Evans (2004), Kumar *et al.* (2004)
		Stimulate fibroblast to contract collagen matrix	Kumar *et al.* (2004), Grazul-Bliska *et al.* (2003),
		Modulate collagen and collagenase expression	Diegelmann and Evans (2004)

TGF β3	Macrophages	Antiscarring	Lorenz and Longaker (2008), Tyrone *et al.* (2000), Singer and Clark (1999)
TGF α	Macrophages, Platelets, keratinocytes, Fibroblast	Stimulates proliferation of fibroblast and epithelial cells	Kondo and Ishida (2010), Lorenz and Longaker (2008), Lawrence and Diegelmann (1994), Martin *et al.* (1992)
		Angiogenic factor	Bennett and Schultz (1993), Martin *et al.* (1992)
Platelet derived growth factor (PDGF)	Plaletes, fibroblasts, macrophages, endothelial cells, vascular smooth muscle cells, epidermal cells	Chemoattractant for fibroblast, neutrophils, monocytes, smooth muscle cells, lymphocytes	Lorenz and Longaker (2008), Enoch and Leaper (2007), Kumar *et al.* (2004), Diegelmann and Evans (2004), Grazul-Bliska *et al.* (2003), Monaco and Lawrence (2003), Halloran and Slavin (2002), Singer and Clark (1999), Walsh (1998), Lawrence and Diegelmann (1994), Martin *et al.* (1992), Lynch *et al.* (1989)
		Stimulate mitogenic for fibroblast and smooth muscle cells	Enoch and Leaper (2007), Diegelmann and Evans (2004), Grazul-Bliska (2003), Walsh (1998), Bennett and Schultz (1993), Lynch *et al.* (1989)
		Monocyte maturation	Kumar *et al.* (2004)
		Potentiates VEGF production	Kumar *et al.* (2004)
		Stimulate MMP production by fibroblast	Kumar *et al.* (2004), Lynch *et al.* (1989)
		Stimulates myofibroblast to contract matrix collagen	Lorenz and Longaker (2008), Kumar *et al.* (2004)
		Induce fibroblast proliferation	Enoch and Leaper (2007), Lorenz and Longaker (2008), Singer and Clark (1999), Witte and Barbul (1997), Lawrence and

			Diegelmann (1994)
		Stimulates collagen synthesis	Enoch and Leaper (2007), Monaco and Lawrence (2003), Martin *et al.* (1992)
		Increase TGF-β and MCP-1 synthesis	Halloran and Slavin (2002)
		Increase integrin expression	Halloran and Slavin (2002)
Epidermal Growth Factor (EGF)	Platelets, macrophages, keratinocytes	Increase epithelialisation and promotes migration of keratinocytes	Grazul-Bliska *et al.* (2003), Lawrence and Diegelmann (1994), Bennett and Schultz (1993)
		Increase granulation tissue	Lawrence and Diegelmann (1994), Lynch *et al.* (1989)
		Increase fibroblast proliferation	Witte and Barbul (1997), Lawrence and Diegelmann (1994), Bennett and Schultz (1993), Martin *et al.* (1992)
		Stimulates angiogenesis	Lawrence and Diegelmann (1994), Martin *et al.* (1992)
		Stimulates collagenase activity	Lawrence and Diegelmann (1994)
		Chemoattractant for fibroblast, endothelial cells	Lorenz and Longaker (2008), Lawrence and Diegelmann (1994), Martin *et al.* (1992)
basic Fibroblast Growth factor (bFGF)	Macrophages, fibroblast, endothelial cells, keratinocytes, vascular smooth muscle cells, brain, pituitary and mast cells	Angiogenic factor	Kondo and Ishida (2010), Lorenz and Longaker (2008), Enoch and Leaper (2007), Oberyszyn (2007), Grazul-Bliska *et al.* (2003), Monaco and Lawrence (2003), Cockbill (2002), Halloran and Slavin (2002), Prathiba and Gupta (2000), Tonnesen *et al.* (2000), Ortega *et al.* (1998), Singer and Clark (1999), Lawrence and Diegelmann (1994),

			Martin *et al.* (1992), Lynch, *et al.* (1989), Montesano *et al.* (1986)
		Increase proliferation of cappilary endothelial cells, keratinocytes, epithelial cells, fibroblasts	Lorenz and Longaker (2008), Enoch and Leaper (2007), Grazul-Bliska *et al.* (2003), Cockbill (2002), Singer and Clark (1999), ,Lawrence and Diegelmann (1994), Tsuboi and Rifkin (1990)
		Chemoattractant for endothelial cells, fibroblasts, and keratinocytes	Lorenz and Longaker (2008), Grazul-Bliska (2003), Halloran and Slavin (2002), Ortega (1998), Tsuboi and Rifkin (1990)
		Stimulates collagen, fibronectin, and proteoglycan synthesis	Enoch and Leaper (2007), Oberyszyn (2007), Grazul-Bliska *et al.* (2003), Lynch, *et al.* (1989)
		Neurotrophic factor	Grazul-Bliska *et al.* (2003), Ortega (1998), Bennett and Schultz (1993)
Keratinocytes growth Factor (KGF)	Fibroblasts	Stimulates keratinocytes and fibroblasts motility	Lorenz and Longaker (2008), Enoch and Leaper (2007), Kumar *et al.* (2004), Grazul-Bliska *et al.* (2003), Singer and Clark (1999)
		Increase proliferation of keratinocytes	Lorenz and Longaker (2008), Enoch and Leaper (2007), Werner *et al.* (2007), Grazul-Bliska *et al.* (2003), Cockbill (2002), Singer and Clark (1999), Lawrence and Diegelmann (1994), Bennet and Schultz (1993), Martin *et*

			al. (1992)
Vascular Endothelial Growth factor (VEGF)	Platelets, macrophages, fibroblasts, keartinocytes, smooth muscle cells, neutrophils, mast cells	Angiogenic factor	Kondo and Ishida (2010), Bao *et al.* (2009), Lorenz and Longaker (2008), Enoch and Leaper (2007), Oberyszyn (2007), Diegelmann and Evans (2004), Kumar *et al.* (2004), Grazul-Bliska *et al.* (2003), Halloran and Slavin (2002), Tonnesen *et al.* (2000), Singer and Clark (1999)
		Chemoattractant	Halloran and Slavin (2002), Bao *et al.* (2009)
		Induces procoagulant factors in endothelial cells	Bao *et al.* (2009)
		Induces endothelial cells migration	Bao *et al.* (2009)
		Increase proliferation of endothelial cells	Bao *et al.* (2009), Bennett and Schultz (1993)
Insulin -like Growth Factor (IGF)	Liver, fibroblasts, epidermal cells	Stimulates cellular growth and differentiation	Lorenz and Longaker (2008), Enoch and Leaper (2007), Grazul-Bliska *et al.* (2003), Walsh (1998), Singer and Clark (1999), Witte and Barbul (1997),
		Stimulates collagen synthesis by fibroblast	Lorenz and Longaker (2008), Enoch and Leaper (2007), Lawrence and Diegelmann (1994)
		Increase re-epithelialisation and granulation tissue formation	Singer and Clark (1999)
		Stimulates synthesis of sulphates proteoglycans	Enoch and Leaper (2007), Lawrence and Diegelmann (1994)