In vitro BIOACTIVITIES OF *Tabernaemontana divaricata* AND *Tabernaemontana divaricata* **'Flore Pleno' VARIANT**

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In vitro BIOACTIVITIES OF *Tabernaemontana divaricata* AND *Tabernaemontana divaricata* 'Flore Pleno' VARIANT

ABSTRACT

Two *Tabernaemontana* species, *Tabernaemontana divaricata* (TD) and *Tabernaemontana divaricata* 'Flore Pleno' variant (TDF) were chosen to be studied for their bioactivities in relation to wound healing properties. Methanol (M) and aqueous (A) extracts of these *Tabernaemontana* species were used in this study for wound healing potential assessment on adult normal human dermal fibroblasts (HDFa) cells, total phenolic contents (TPC), antioxidant potential, protective effects against hydrogen peroxide (H_2O_2) and anti-inflammatory properties by means of nitric oxide (NO) production from RAW264.7 murine macrophage cells. TD-A extract reported the highest wound closure (58.50%) followed by TDF-A extract (53.54%) with the highest total phenolic content (31.96 mg GAE/g). The free radical scavenging activities carried out by ABTS and DPPH assay showed that TDF-A extract was able to exhibit the highest antioxidant values at IC_{50} = 1.11 µg/ml and IC_{50} = 2.44 µg/ml respectively compared to the other extracts. Reducing activity on TDF-A extract assessed by FRAP assay also reported high value of [18.07 mM Fe (II)/g] with other extracts. Pre-treatment and post-treatment assessment showed at 25 µg/ml of TDF-M extract concentration were able to provide protectivity towards HDFa cells against H_2O_2 with cell viability at 93.92% and 89.69% respectively. At 500 µg/ml of TD-A and TDF-A extract concentration, the extracts were able to inhibit NO production by RAW264.7 murine macrophage cells at 5.22 µM and 5.11 µM respectively. Based on these findings, it is shown the two *Tabernaemontana* species studied exhibit multiple bioactivities that may contribute to the wound healing process in general.

Keywords: *Tabernaemontana divaricata*, *Tabernaemontana divaricata* 'Flore Pleno', wound healing, antioxidant, protectivity, anti-inflammatory.

BIOAKTIVITI *in vitro Tabernaemontana divaricata* DAN

***Tabernaemontana divaricata* VARIAN 'Flore Pleno'**

ABSTRAK

Dua spesies *Tabernaemontana*, *Tabernaemontana divaricata* (TD) dan *Tabernaemontana divaricata* varian 'Flore Pleno' (TDF) dipilih untuk mengkaji bioaktiviti-bioaktiviti berkaitan sifat-sifat penyembuhan luka. Ekstrak methanol (M) dan akueus (A) spesies *Tabernaemontana* telah digunakan di dalam kajian ini bagi penilaian potensi penyembuhan luka ke atas sel-sel fibroblast biasa manusia (HDFa), jumlah kandungan fenolik (TPC), potensi antioksida, kesan perlindungan kepada sel-sel HDFa terhadap hidrogen peroksida (H_2O_2) dan sifat anti-radang melalui pengeluaran nitrik oksida (NO) daripada sel-sel murin makrofaj RAW264.7. Ekstrak TD-A melaporkan penutupan luka tertinggi (58.50%) diikuti dengan ekstrak TDF-A (53.54%) yang melaporkan jumlah kandungan fenolik tertinggi (31.96 mg GAE/g). Aktiviti pengaut radikal bebas yang diuji melalui asei ABTS dan DPPH telah menunjukkan ekstrak TDF-A mampu menghasilkan nilai antioksidan tertinggi masing-masing pada IC_{50} = 1.11 µg/ml dan IC_{50} = 2.44 µg/ml berbanding ekstrak lain. Aktiviti pengurangan oksida terhadap ekstrak TDF-A dinilai melalui asei FRAP juga melaporkan nilai yang tinggi [18.07 mM Fe (II)/g] bersama ekstrak lain. Penilaian pra-rawatan dan pasca-rawatan menunjukkan pada kepekatan 25 µg/ml, ekstrak TDF-M dapat memberikan perlindungan kepada sel-sel HDFa terhadap H_2O_2 dengan daya maju sel masing-masing pada 93.92% dan 89.69%. Pada kepekatan ekstrak 500 µg/ml TD-A dan TDF-A, ekstrak-ekstrak dapat menghalang pengeluaran NO oleh sel-sel murin makrofaj RAW264.7 masing-masing pada 5.22 µM dan 5.11 µM. Berdasarkan penemuan ini, ditunjukkan bahawa dua spesies *Tabernaemontana* yang dikaji menunjukkan banyak bioaktiviti yang dapat menyumbang kepada proses penyembuhan luka secara umum.

Kata kunci: *Tabernaemontana divaricata*, *Tabernaemontana divaricata* ‘Flore Pleno’, penyembuhan luka, antioksida, perlindungan, anti-radang.

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LIST OF SYMBOLS AND ABBREVIATIONS

ºC	:	Degree Celcius
%	:	Percentage
±	:	Plus-Minus
µm	:	Micrometer
µM	:	Micromole
µg/ml	:	Microgram per mililiter
µM/ml	:	Micromole per mililiter
ANOVA	:	Analysis of Variance
ATCC	:	American Type Culture Collection
ABTS	:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
BHA	:	Butylated hydroxyanisole
cells/ml	:	Cells per mililiter
cm	:	Centimeter
CO_2	:	Carbon dioxide
DMEM	:	Dulbecco's Modified Eagle's Medium
DMSO	:	Dimethyl sulfoxide
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
FBS	:	Foetal Bovine Serum

F-C	:	Folin-Ciocalteau
Fe	:	Ferric
FRAP	:	Ferric Reducing Antioxidant Power
g	:	Gram
GAE	:	Gallic Acid Equivalent
h	:	Hour
HDFa	:	Human Dermal Fibroblast, Adult
H_2O_2	:	Hydrogen Peroxide
IBM	:	International Business Machines
IC_{50}	:	Half Maximal Inhibitory Concentration
kg	:	Kilograms
mg/ml	:	Miligram per mililiter
mM	:	Millimole
mm	:	Millimeter
nm	:	Nanometer
nm/h	:	Nanometer per hour
NRU	:	Neutral Red Uptake
NO	:	Nitric Oxide
OD	:	Optical Density
pH	:	Potential of Hydrogen

r	:	Pearson's Correlation Coefficient
SD	:	Standard Deviation
SPSS	:	Statistical Package for the Social Sciences
TPC	:	Total Phenolic Content
TPTZ	:	2,4,6-tri-(pyridyl)-s-triazine
v/v	:	Volume per volume
w/v	:	Weight per volume

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CHAPTER 1: INTRODUCTION

1.1 Research Background

Medicinal plants have always been the base and foundation in traditional medicine for more than thousands of years which are used by the Greeks, Chinese, Indians, Arabs, and Pakistanis (Khan, 2014), by providing possible cures for diseases back then when modern medicine was not yet discovered. Medicinal plants are a reservoir of ingredients, containing rich in resources for the development and production of drugs, apart from playing an important role in multiple and different cultures around the world (Singh, 2015). According to the earliest records found inscribed on tablets in cuneiform carved by the Mesopotamians back in 2,600 BC, the traditional application of plant extracts include the oils of *Commiphora* species (Myrrh), *Cedrus* species (Cedar), *Glycyrrhiza glabra* (Licorice), *Papaver somniferum* (Poppy juice) and *Cupresses sempervirens* (Cypress) are a few of the plant species mentioned that is still widely used until today to treat colds, coughs, inflammations, and parasitic infections (Dar *et al*., 2017).

Wounds are formed mainly on the skin when it is cut, opened, or contused caused by a blunt force trauma (Qureshi, 2015). The common types of wounds include laceration caused by a straight or uneven skin tear, abrasion occurring by scraping the skin against a rough surface, bite or puncture wound caused by sharp object penetration and burn wound caused by thermal injury (Worster *et al*, 2015). Wound healing is a four-phase physiological mechanism that is carried out by the body by restoring and replacing function to damaged tissues. The four main phases of wound healing are (i) haemostasis, (ii) inflammation, (iii) proliferation (granulation and contraction); and (iv) remodeling (maturation phase) (Flanagan, 2000; Orsted *et al*., 2011).

After immediate wound formation, fibroblast proliferation is activated through stimulation by growth factors which undergoes migration to the wound site. Usually on the third day, fibroblasts are abundant in the wound site, secreting extracellular matrix (ECM) proteins (fibronectins, hyaluronan and proteoglycans) and subsequently producing collagen and fibronectin leading to granulation. Once the fibroblasts are abundant enough in number, this leads to the conversion of fibroblasts into myofibroblasts which plays an important role in wound contraction (Harper *et al.*, 2014). The main event in wound healing of the connective tissue is the differentiation of fibroblasts into myofibroblasts. In wounds, fibroblasts are often activated and differentiate into myofibroblasts which contracts, aids in the wound size reduction and secretes ECM proteins. Myofibroblasts can produce a contractile force which enables it to close an open wound by facilitating the edges of a wound to a close (Li & Wang, 2011).

While wound healing is a continuous and ongoing process, there are issues circulating in relation to its effectiveness over time. Firstly, are not all wounds showing healing challenges are similar. Secondly, no two patients can heal and potential of recovery at the same time. Thirdly, the healing of wound might be affected according to the patients' intentions and desires while recovering. Fourthly, not all wounds are able to heal at a similar rate to other wounds. The fifth issue is healing requires time and patience as it undergoes a series of phases to fully recover. Sixth, different type of wounds requires specific treatments that can provide the maximum healing outcome. The final issue is that the determination of proximate prevention measures to ensure total wound healing is already defined. However, the stubbornness of patients' in neglecting these measures result in a recurring wound and longer time to fully heal (Strauss *et al*., n.d.).

There are several studies carried out on wound healing using plant extracts from all around the world. Plants have long been used as remedies to treat diseases and

particularly wounds due to the respective properties it contains according to different plant types. Numerous plants are used by different tribes in different countries to treat wounds and burns as they promote healing and tissue regeneration through specific mechanisms (Thakur *et al.*, 2011). A common example of plant extract used to treat wound healing is *Aloe vera*. This plant has firstly been used by the Egyptians to relieve wounds, burn wounds and infections before the Greeks, Spanish and Africans applied the plant extracts for different purposes. *A. vera* contains a very thick mucilage tissue (aloe gel) located within the leaves that is extracted and administered for various medical applications (Hashemi *et al.*, 2015). *A. vera* provides a solution to treat minor burns, incision wounds and sunburns whereby the juice and aqueous extracts from the leaves contain healing properties in relation to wound healing. Another medicinal plant is *Gingko biloba*, where it has been tested out on male rats by promoting wound closure at a faster rate. Besides wound healing, *G. biloba* extracts are used by the Chinese as a traditional anti-inflammatory and antiallergenic Chinese medicine. In addition, *Centella asiatica* is also another medicinal herb which has been formulated into ointment, cream and gels from the aqueous extracts obtained (Sabale *et al*., 2012). *C. asiatica* is also effective for the treatment of systemic scleroderma, abnormal scarring, and keloids due to the asiaticoside, asiatic acid and madecassic acid contained in its extracts (Pawar & Toppo, 2012).

Tabernaemontana species comes from the family Apocynaceae. The family Apocynaceae make up 3,700 species in 424 genera (Endress & Bruyns, 2000). It is a tropical and subtropical plant that can often be found in Africa, America, and Asia (Silveira *et al*., 2017). *Tabernaemontana* species has long be used in traditional medicine as it possesses cytotoxicity, antihelminthic, analgesic and antinematodal activities (Abubakar & Loh, 2016). *Tabernaemontana divaricata* is a green plant shrub, with a

height of 54 cm has shiny leaves and crepe jasmines as flowers. In tropical countries, it is a garden plant that is commonly used due to its abundance of alkaloids. A study was carried out by Selvakumar & Kumar (2015) to determine the antiproliferative properties of *T. divaricata* against HEP2 cell line and Vero cell lines. In a separate study, the leaves of *T. divaricata* are used as an antifungal against the fungal pathogen *Candida albicans* that can infect humans (Wankhede *et al.*, 2013). In another study, antioxidant and cytotoxic properties obtained from the methanolic extracts of leaves from *T. divaricata* were carried out (Rumzhum *et al.*, 2012). Numerous studies have already been carried out on the pharmacological properties of *T. divaricata*, its medicinal use is not only limited to certain diseases but due to its antimicrobial properties it is able to combat against diseases including syphilis, leprosy, gonorrhoea, dysentery, diarrhoea, malaria, and as an antiparasitic against worms (van Beek *et al.*, 1984; Pratchayasakul *et al.*, 2008). *Tabernaemontana corymbosa* is another species of *Tabernaemontana*, where a phytochemical analysis was done on its leaves to determine the phenolic compounds using high performance liquid chromatography (HPLC) (Nayeem *et al.*, 2014).

In this study, *T. divaricata* and *T. divaricata* 'Flore Pleno' variant were investigated for their wound healing effects and other biological activities in relation with the wound healing process such as antioxidant activity, anti-inflammatory and protective effects. In addition, this study will prove the traditional claims of *Tabernaemontana* species in wound management and might contribute to its development as a potential wound healing agent. This study aims to evaluate the biological activities in relation with wound healing processes of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant extracts.

1.2 Objectives of Study

The specific objectives of this present study are:

i. To evaluate the wound healing potential of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant extracts using *in vitro* model on normal human dermal fibroblast (HDFa) cells.

ii. To evaluate total phenolic content and antioxidant activity of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant extracts.

iii. To investigate the protective effects of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant extracts against hydroxyl radical.

iv. To determine anti-inflammatory effects of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant extracts associated with its wound healing properties.

CHAPTER 2: LITERATURE REVIEW

2.1 Skin Wound

2.1.1 Introduction to the Anatomy of the Skin

The skin is the largest organ in the human body. It mainly functions as (i) an external stimuli, (ii) a physical barrier between the body and the external environment by reducing any chemical penetration that may come in contact with the skin and prevent harmful microorganisms from breaching into the body; (iii) homeostasis maintenance by regulating body temperature and preventing water loss and (iv) vitamin D synthesis. The skin is composed of three layers: the epidermis, dermis and hypodermis (subcutaneous fat) (Figure 2.1) (Khavkin & Ellis, 2011; McLafferty *et al.*, 2012).

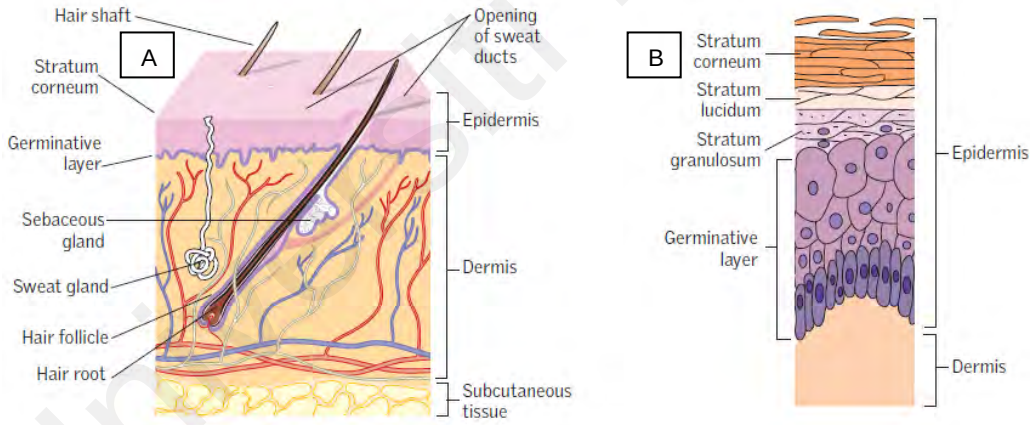

Figure 2.1: (A) Skin cross section showing the three main layers of the skin; (i) epidermis, (ii) dermis and (iii) subcutaneous tissue with its components and (B) close-up cross section of the epidermis and dermis (McLafferty *et al.*, 2012).

The epidermis (Figure 2.2) makes up the outermost layer of the skin, is a layer which continuously grows and renews the skin over time. The epidermis is made up of keratinocytes that synthesizes keratin which plays an important protective role. The dermis is located between the epidermis and the subcutaneous fat. The main cellular

component of the dermis are the fibroblast cells which include the fibrillar, collagen and elastic fibres (Ribeiro *et al.*, 2017).

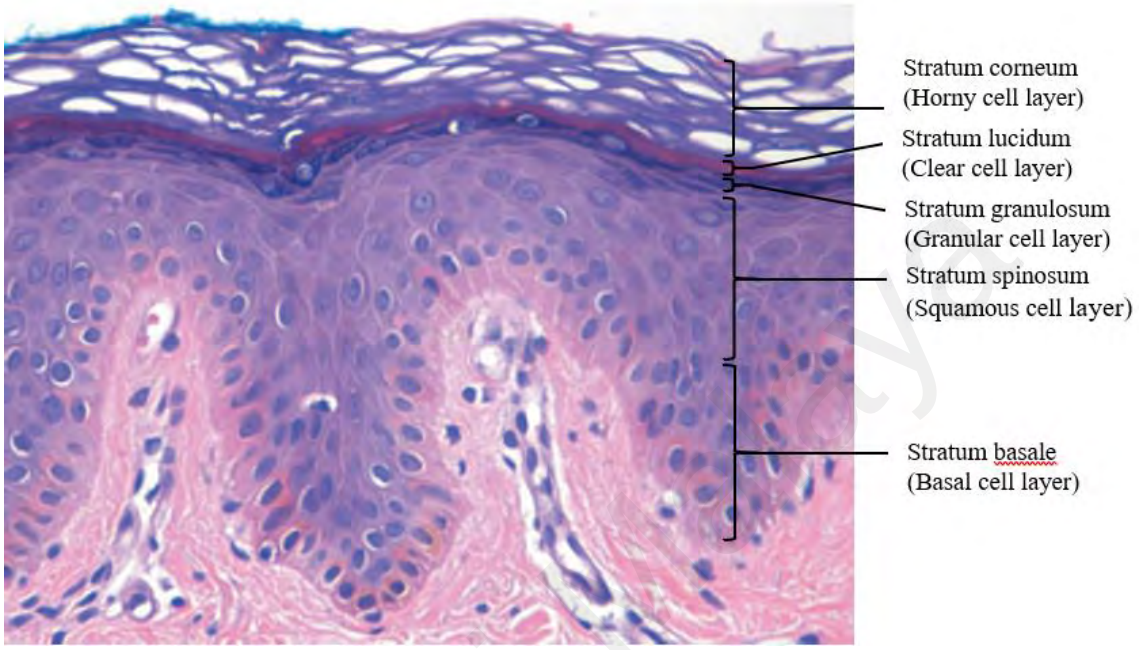

Figure 2.2: Epidermis of the human skin. Enucleated cells is comprised of (from bottom to top) stratum basale (basal cell layer), stratum spinosum (squamous cell layer), stratum lucidum (clear layer) and stratum granulosum (granular cell layer) respectively while anucleated cells of the epidermis called stratum corneum (cornified or horny cell layer) is accumulated on the surface of the skin and is mainly composed of dead cells. Magnification 200× (Ribeiro *et al.*, 2017).

The dermis is located below the epidermis, consisting of a unified system of fibrous, filamentous connective tissue that makes up the nerve and vascular network, epidermally derived appendages, fibroblasts, macrophages and mast cells. It is also the thickest part of the skin which makes its pliable, elastic and provides tensile strength for the skin. In addition, the dermis plays a major role by protecting the body from injuries, reduces water loss, regulates heat dissipation and houses sensation receptors for stimuli (Kolarsick *et al.*, 2011).

The hypodermis is the final layer of the skin located just below the dermis and underlying muscle and is made up of adipocytes. Besides protecting the body from

mechanical harm, it functions well both as insulation and acts as an energy reserve. Adipocytes within the hypodermis varies in thickness among individuals and represents the nutritional stature of that individual (Khavkin & Ellis, 2011).

Keratinocytes make up the most cells within the epidermis layer of the skin. Keratinization is the process involved when keratinocytes move from the basal layer of the epidermis towards the surface of the skin. The basal cell proliferation (germinating layer) occurring under the epidermis provides a new layer for the external part of the epidermis, making it a continuous process over time to rise up towards the skin surface with other non-keratinocyte cells of the epidermis (Chu, 2008; Kolarsick *et al*., 2011).

Other non-keratinocyte cells that make up the cell population within the epidermis also include melanocytes, Merkel cells and Langerhans cells. The epidermis itself can be divided into four more layers which is arranged according to the morphology of the cells present within that layer. These layers comprise of the stratum basale (basal cell layer), stratum spinosum (squamous cell layer), stratum granulosum (granular cell layer) and stratum corneum (cornified or horny cell layer) (James *et al.*, 2006; Kolarsick *et al.*, 2011).

Another layer called stratum lucidum (clear layer) placed between stratum granulosum and stratum corneum is made up of three to five layers of flattened, translucent and dead keratinocytes. This layer is most common in areas such as the palms and feet soles where the skin is thick (McLafferty *et al.*, 2012). In addition, cellular division rate can be affected by certain conditions such as wounding. At least 14 days is required for cells from the basal layer to the cornified layer and another 14 days to reach the skin surface (Chu, 2008; Kolarsick *et al.*, 2011; Ribeiro *et al.*, 2017).

Melanocytes are pigment-synthesizing and dendritic cells which are derived from the neural crest and is embedded within the basal layer of the skin. While it does not play

a direct part with keratinocytes, their role is to produce melanin and transfer it to keratinocytes (Chu, 2008; Kolarsick *et al.*, 2011). Merkel cells are mechanoreceptors conveniently placed at locations within the skin with high touch sensitivity such as the lips, oral cavity area and fingertips. Merkel cells are activated by external stimulus, causing them to secrete chemical signals by inducing action potential in the afferent neuron to send a signal to the human brain. Langerhans cells emerge from the bone marrow and are associated in multiple series of responses specifically involving T-cells. From the bone marrow, these cells move upwards to the epidermis in its embryonic stages and pursue to distribute and repopulate the epidermis continuously over time (Kolarsick *et al.*, 2011).

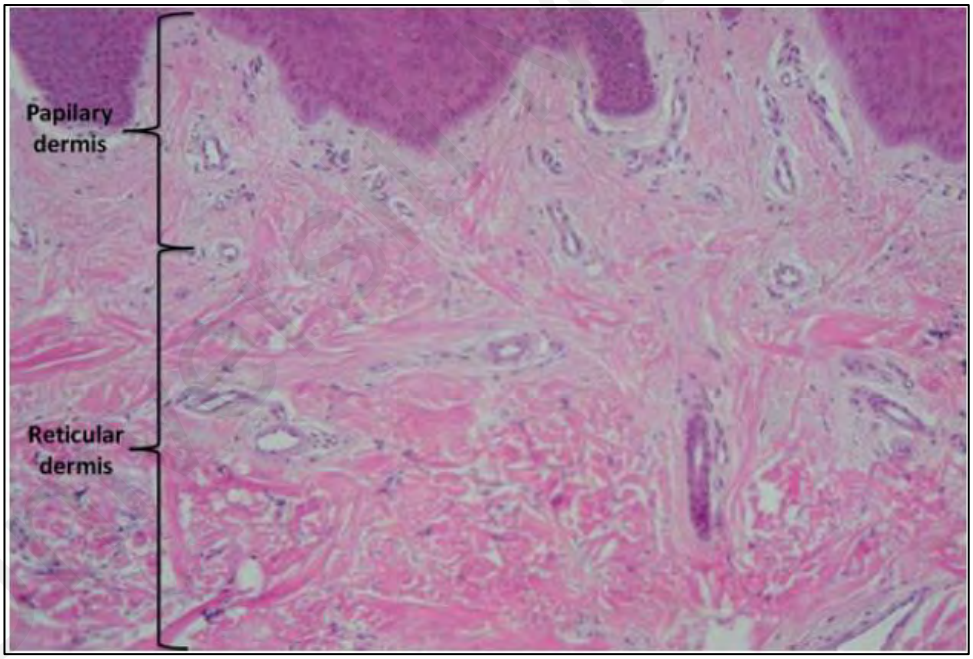

Figure 2.3: Dermis of the human skin. Fibroblasts are evenly distributed within the dermis due to the presence of intercellular substances and is meshed together with a substantial amount of collagen fibres (pink) and ground substance present. Magnification 100× (Ribeiro *et al.*, 2017).

Based on Figure 2.3, the dermis can be distinctly divided into an upper papillary dermis and a lower reticular dermis. The papillary dermis is located just below the dermal-epidermal junction of forming conic upward projections which increases the

dermal-epidermal interface area and provides a better attachment of the dermis and epidermis. The papillary dermis differs from the reticular dermis in terms of its placement within the skin and the components it holds. The papillary dermis is made up of a loose assortment of collagen, elastic fibres, fibrocytes, blood vessels and nerve endings whereas the reticular dermis has a more compact placement of collagen fibres, much more thick elastic fibres, vascular and nerve networks (Khavkin & Ellis, 2011). Collagen is the main component within the dermis which has stress-resistant properties while collagen fibres maintain elasticity of the skin (James *et al.*, 2006). Collagen fibres also act as a support system for the integument together with limiting stretching of the skin while elastic fibres plays an important role in retaining the skin back to its original area once force applied to that part of skin is removed (Ribeiro *et al.*, 2017). The hypodermis is the layer located just below the dermis, where the skin lies on this surface and at the same time attaches the dermis to the muscles and bones underlying beneath (McLafferty *et al.*, 2012). The hypodermis houses abundant adipose tissues when great in number can form clusters or masses of cells to act as a cushion for the skin (Monteiro-Riviere, 2010).

2.1.2 Types of Skin Wound

Wounds are formed mainly on the skin when it is cut, opened, or contused caused by a blunt force trauma (Qureshi, 2015). The common types of wound include (i) laceration caused by a straight or uneven skin tear, (ii) abrasion occurring by scraping the skin against a rough surface, (iii) bite or puncture wound caused by sharp object penetration and (iv) burn wound caused by thermal injury (Worster *et al.*, 2015).

2.2 Wound Healing Process

Wound healing is a four-phase physiological mechanism that is carried out by the body by restoring and replacing function to damaged tissues. The four main phases of wound healing are (i) haemostasis, (ii) inflammation, (iii) proliferation (granulation and contraction) and (iv) remodeling (maturation phase) (Flanagan, 2000; Orsted *et al.*, 2011; Schultz *et al.*, 2011).

Wound healing is an instant, continuous and overlapping process. The process in wound healing is a time-dependent event integrated with intrinsic and extrinsic factors that may affect the condition and healing of the individual wound over time. In addition, wound healing process involves a range of cells, main cells being fibroblasts and keratinocytes. These cells are regulated by a plethora of growth factors and cytokines that may accelerate or decelerate the healing progress of the wound. In addition, wound healing is also dependent on the type of wound injury and the characteristic of the wound which differs from one person to another in relation to their age and lifestyle (Guo & Dipetrio, 2010). Table 2.1 and Figure 2.4 show the four phases involved in the wound healing.

Table 2.1: Phases of wound healing.

Phases	Time of Action / Duration	Cellular Events
Haemostasis	Seconds to minutes upon injury	-Vascular constriction
		- Platelet aggregation
		- Degranulation
		- Fibrin formation
Inflammation	24 hours upon injury	- Neutrophil infiltration
		- Monocyte infiltration and differentiation to macrophage
		- Lymphocyte infiltration
Proliferation	Three to ten days upon injury	- Re-epithelialization
		- Angiogenesis
		- Collagen synthesis
		- ECM formation
Remodeling	Two to three weeks upon injury	- Collagen remodeling
		- Vascular maturation and regression

ECM = Extracellular matrix. [Adapted from Guo & Dipetrio, (2010)]

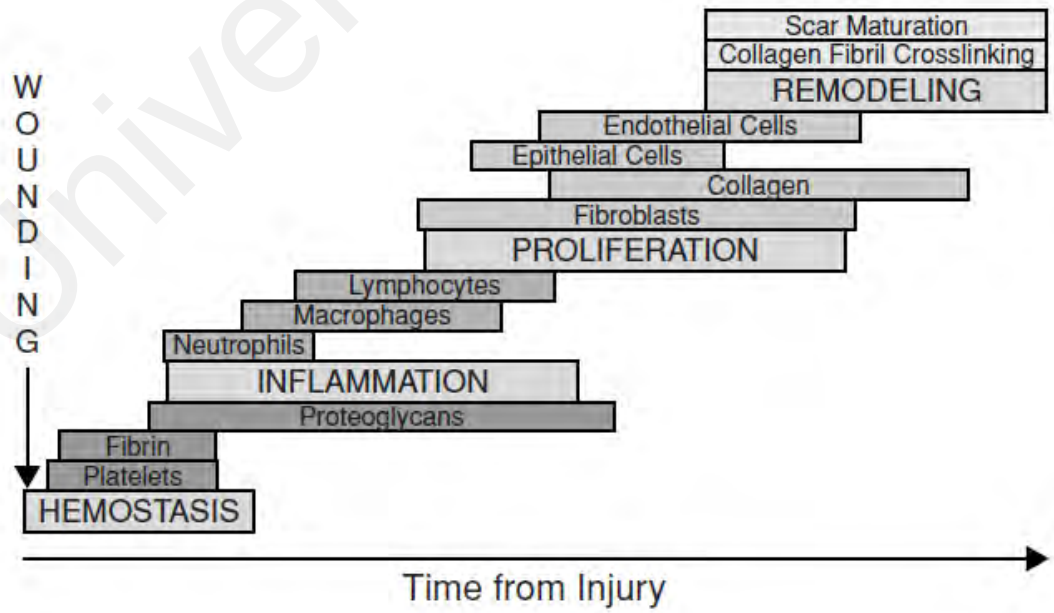

Figure 2.4: Wound healing cascade. Cellular components and synthesized products during the four major wound healing phases: Haemostasis, inflammation, proliferation and remodeling leading up to full scar maturation (Schultz *et al.*, 2011).

Presence of free radicals and oxidative reactions play a vital role in accelerating wound healing. If the production of reactive oxygen species (ROS) in abnormally high, it can quicken inflammation and accelerate cellular damage caused by oxidative stress. This would lead to a delayed in the wound healing process. The inflammatory phase plays a vital role in wound healing as nitric oxide (NO) production promotes vasodilation followed by cell migration. Limiting NO production can be therapeutic in treating and healing wounds, as too much NO production can impair the wound healing process. Therefore, studies carried out focusing on antioxidant, anti-inflammatory and ROS elimination could prove beneficial to treating wounds (Chaniad *et al.*, 2020).

2.2.1 Haemostasis

Wound healing phases are led by haemostasis which occurs within seconds to minutes upon wound induction (Gantwerker & Hom, 2012), followed by provisional wound matrix formation before initiation of inflammation phase. Haemostasis may occur up to the three days, initiated by a series of wound healing steps directly upon wound formation. During haemostasis, a primary clot also forms within minutes to hours before entering inflammatory phase (Busse, 2016).

The main component involved during this phase of wound healing are platelets. They are the first cells to arrive upon wound formation and they function by forming a blood clot surrounding the wound area to begin a process involving the construction of an ECM to ensure the delivery of important components to the surface of a wound which are crucial to the healing of a wound (Busse, 2016).

Next are neutrophils and macrophages, where its primary function is to release enzymes for the removal of bacteria and debris present within the wound and assist neutrophils in the removal of debris carried out by its phagocytic capabilities. In addition, macrophages also produce NO and assist neutrophils in maintaining the antimicrobial activities occurring within the wound. Ultimately, macrophages induce collagen production and neovascularization through the release of growth factors and cytokines (Busse, 2016).

2.2.2 Inflammation

The second phase in wound healing occurs within the first 24 hours and can extend up to two weeks in acute wounds and longer in chronic wounds (Schultz *et al.*, 2011). The inflammatory response is important for supplying growth factors and cytokine signals for cell and tissue migration which plays a major role in repair mechanisms in humans (Reinke & Sorg, 2012). Inflammation is designated when very distinct signals are seen and felt such as rubor (redness), calor (heat), tumor (swelling) and dolor (pain) is obvious surrounding the area of the wound (Schultz *et al.*, 2011).

Cells involved in this phase are neutrophils, monocytes and macrophages (Schultz *et al.*, 2011) which are regulated by chemokines, vasodilation and increment of blood vessel permeability (Delavary *et al*., 2011). These cells act on the wound by clearing away all impurities. At the same time, those cells release cytokines involved in inflammatory stage of wound healing such as interleukin-1 (IL-1), interleukin-6 (IL-6), interleukin-8 (IL-8), tumor necrosis factor-α (TNF-α) and growth factors including platelet-derived growth factor (PDGF), transforming growth factor-β (TGF-β), transforming growth factor-α (TGF-α), fibroblast growth factor (FGF) and insulin-like growth factor (IGF-1). Many of these cells are involved in assembling and the

mobilization of fibroblasts and epithelial cells for proliferation stage in wound healing (Schultz *et al.*, 2011). Roughly after three days of wound formation, monocytes travel to the wound and promote wound healing by differentiating into macrophages which play a vital role which promote the inflammatory-proliferative transition of phase leading up to the proliferation phase in wound healing (Landén *et al.*, 2016).

2.2.3 Proliferation

The third phase comprises of multiple processes such as re-epithelialization, angiogenesis, granulation tissue formation, and collagen deposition to promote healing of the wound (Sinno & Prakash, 2013). Around three to ten days after wounding, proliferation stage of wound healing takes place. Synthesis of collagen, fibronectin and other substances are regulated by cytokines such as interferon-gamma (IFN-γ) and TGF-β for wound healing to be carried out by fibroblasts. The migration of fibroblasts serve as a base for the reduction and eventually closure of gaps within the tissue and finally providing enough support for the wound to hold itself (Reinke & Sorg, 2012).

Furthermore, fibroblasts are mainly involved in granulation tissue formation by migrating from the dermis to the wound site via signals released by certain cytokines and growth factors such as platelet-derived growth factors (PDGF) and TGF-β, produced solely by platelets and macrophages. Conversely, if the wound condition prolongs, fibroblasts are also able to surface from fibrocytes, a bone marrow-derived cell. Wandering fibrocytes can travel to wound affected areas to promote healing by supplying fibroblasts alongside producing cytokines and growth factors and at the same time promoting angiogenesis (Landén *et al.*, 2016).

Once the inflammatory response is done, re-epithelialization occurs immediately after this healing process. This is the reconstitution of cells to cover the epidermis on the wounded area of the skin. The keratinocyte restoration usually occurs within hours upon wound formation by moving along the granulation tissue from both sides until a contact is re-established (Delavary *et al.*, 2011). Re-epithelialization depends on epithelial cell migration from wound edges and the dermis to the wound (Janis *et al.*, 2010).

A series of cells activate re-epithelialization by releasing cytokines and growth factors such as epidermal growth factor (EGF), keratinocyte growth factor (KGF), IGF-1, and nerve growth factor (NGF) (Reinke & Sorg, 2012). Re-epithelialization can also be induced from other signals released during wound formation such as NO production that can only be synthesized by macrophages (Landén *et al.*, 2016). Table 2.2 summarized all the cells involved in wound healing process.

In addition to the increase demand of oxygen and nutrients, reconstruction of the blood vessels and formation of new capillaries in the dermis in wounded areas are important in wound healing (Delavary *et al.*, 2011; Landén *et al.*, 2016). Upon wound formation, not only layers of the skin are damaged but this also includes the blood vessels that run underneath the skin. Therefore, a reconstructive process called angiogenesis is carried out and activated mainly due to wound closure leading to a proximity of damaged blood vessels underlying the dermis (Gantwerker & Hom, 2012).

Angiogenesis is also regulated by a number of cytokines and growth factors which promotes reconstitution of blood vessels within the dermis over time (Gantwerker & Hom, 2012) such as FGF, vascular endothelial growth factor (VEGF), TGF-β, TNF-α, angiogenin, angiotropin and angiopoetin 1 (Sinno & Prakash, 2013). All growth factors and cytokines involved in inflammation and proliferation stage during wound healing is tabulated in Table 2.3.

Table 2.2: Cells involved in wound healing.

Cell Types	Time of Action	Functions
Platelets	Seconds	- Thrombus formation - α granules are a rich source of inflammatory mediators including cytokines (PDGF, TGF-β, FGF, EGF, β-thromboglobulin, platelet factor-4, histamine, serotonin, bradykinin, prostaglandins and thromboxane) - Major initial stimulus for inflammation
Neutrophils	24 hours	- First cells to infiltrate injury site - Phagocytosis of bacteria invading wound site - Removes debris - Releases proteolytic enzymes - Generation of oxygen free radicals - Increases vascular permeability
Keratinocytes	8 hours	- Release of inflammatory mediators - Migration stimulation of neighbouring keratinocytes - Angiogenesis
Monocytes (macrophages)	48–72 hours	- Phagocytose and destroy invading bacteria - Source of inflammatory mediators (cytokines) - Stimulate fibroblast division, collagen synthesis and angiogenesis
Lymphocytes	72–120 hours	- Regulation of proliferative phase in wound healing - May produce cytokines in certain types of wound
Fibroblasts	120 hours	- Synthesize granulation tissue - Produce various components of the ECM, including collagen, fibronectin, hyaluronic acid, proteoglycans - Release of inflammatory mediators

[Adapted from Enoch & Leaper (2008) and Singh *et al.* (2017)]

Table 2.3: Growth factors and cytokines involved in wound healing.

Factors	Sources	Effects
Transforming Growth Factor-α (TGF-α)	- Macrophages - Lymphocytes - Keratinocytes	- Stimulates keratinocyte proliferation - Fibroblast proliferation and migration
Transforming Growth Factor-β (TGF-β)	- Platelets - Lymphocytes - Macrophages - Endothelium - Keratinocytes	- Chemotactic - Stimulates angiogenesis - Stimulation of fibroblast proliferation
Epidermal Growth Factor	-Platelets -Macrophages -Endothelium	- Chemotaxis - Stimulates angiogenesis - Wound contraction - Fibroblast proliferation
Fibroblast Growth Factor (FGF)	- Macrophages - Lymphocytes - Endothelium - Mast cells	- Stimulates angiogenesis - Keratinocyte - Fibroblast proliferation and migration
Keratinocyte Growth Factor (KGF)	- Fibroblasts	- Stimulates keratinocyte migration, differentiation and proliferation
Tumor Necrosis Factor-α (TNF-α)	- Macrophages - Lymphocytes - Mast cells	- Causes fever - Corticotropin-releasing hormone secretion - Suppresses appetite - Stimulates acute-phase responses - Stimulates macrophages and fibroblasts - Angiogenesis
Interleukin-1 (IL-1)	- Macrophages - Lymphocytes - Mast cells	- Causes fever - Stimulates acute-phase response - Stimulates neutrophils - Stimulates macrophages - Stimulates TNF - Stimulates interferon release
Interleukin-2 (IL-2)	- Macrophages - Lymphocytes - Mast cells	- Causes fever - Stimulates macrophages - Lymphocyte differentiation and proliferation
Interleukin-6 (IL-6)	- Macrophages - Lymphocytes - Mast cells	- Causes fever - Stimulates acute-phase response - Upregulation of Toll-like receptors

[Adapted from (Janis *et al.*, 2010)]

Table 2.3, continued.

Factors	Sources	Effects
Interleukin-8 (IL-8)	- Macrophages - Lymphocytes - Mast cells	- Chemotactic - Stimulates neutrophils
Platelet-derived Growth Factor (PDGF)	- Platelets - Macrophages - Endothelium	- Chemotactic - Stimulates fibroblasts - Angiogenesis - Wound contraction
Vascular Endothelial Growth Factor (VEGF)	- Keratinocytes - Hypoxic cells	- Stimulates angiogenesis
Endothelium-Derived Growth Factor (Nitric Oxide)	- Endothelium	- Vasodilation - Antibacterial action - Angiogenesis - Apoptosis

[Adapted from (Janis *et al.*, 2010)]

2.2.4 Remodeling

The remodeling process is the end stage of wound healing, a phase that is regulated continuously by multiple growth factors and numerous cells starting from the initial healing phase to achieve this maturation stage of wound healing (Sinno & Prakash, 2013). Remodeling of the wound normally starts within two to three weeks after the wound formation and may last for up to one year. During this final stage, normal tissue and granulation tissue are re-established and is achieved with increased tensile strength and elasticity through reorganizing, degrading and synthesis of the ECM (de Oliveira Gonzalez *et al.*, 2016). Cell proliferation and synthesis of protein ceases while collagen reconstruction is abundant during this stage. Moreover, nutrient supply decreases, capillaries are reattached and re-established and slowly reduces inflammation, bringing about reduced redness of the wound scar (Gurtner *et al.*, 2008; Delavary *et al.*, 2011).

Granulation tissue maturation leads to a lessen number of capillaries which aggregate together to form larger blood vessels. In addition, collagen type and amount is drastically influenced during this remodeling stage, where type III collagen is produced at a high amount and is succeeded by type I collagen. This affects the tissue tensile strength by reorganization of irregular accumulation of collagen fibres during granulation. However, healed tissue is not as robust as non-wounded tissue of the skin. Over time, collagen is gradually restored and can escalate the tensile strength up to 80% of normal skin tissue (Schultz *et al.*, 2011). One week after wound formation, tensile strength of the wound is only around 3%. An increment to 30% is shown after three weeks and culminated fully at 80% after three months of post-injury (Janis *et al.*, 2010).

Collagen availability increases the tensile strength of the wound by cross-linkage of collagen with the interaction of fibroblasts converted into myofibroblasts. Collagen is repeatedly reduced and synthesized into a more organized and stable manner, leading to an improved tensile strength. However, synthesis of collagen and tensile strength would have improved adequately within 10 days of initial time of wound formation but its composition is only similar to 20% of normal skin. Unlike unwounded skin, wounded or injured skin will never be able to revert back to its original strength and tensile strength, maintaining around only 70% to 80% of its initial strength (Busse, 2016).

Remodeling phase is the definite step in wound healing by ceasing angiogenesis, diminishing blood flow to the wound and slowly reducing metabolic activity within the wound until it permanently ends. In severe injuries, skin components such as hair follicles and sweat glands do not have the ability to be mended and regrown back over time (Reinke & Sorg, 2012). The wound healing phases were summarized in Figure 2.5.

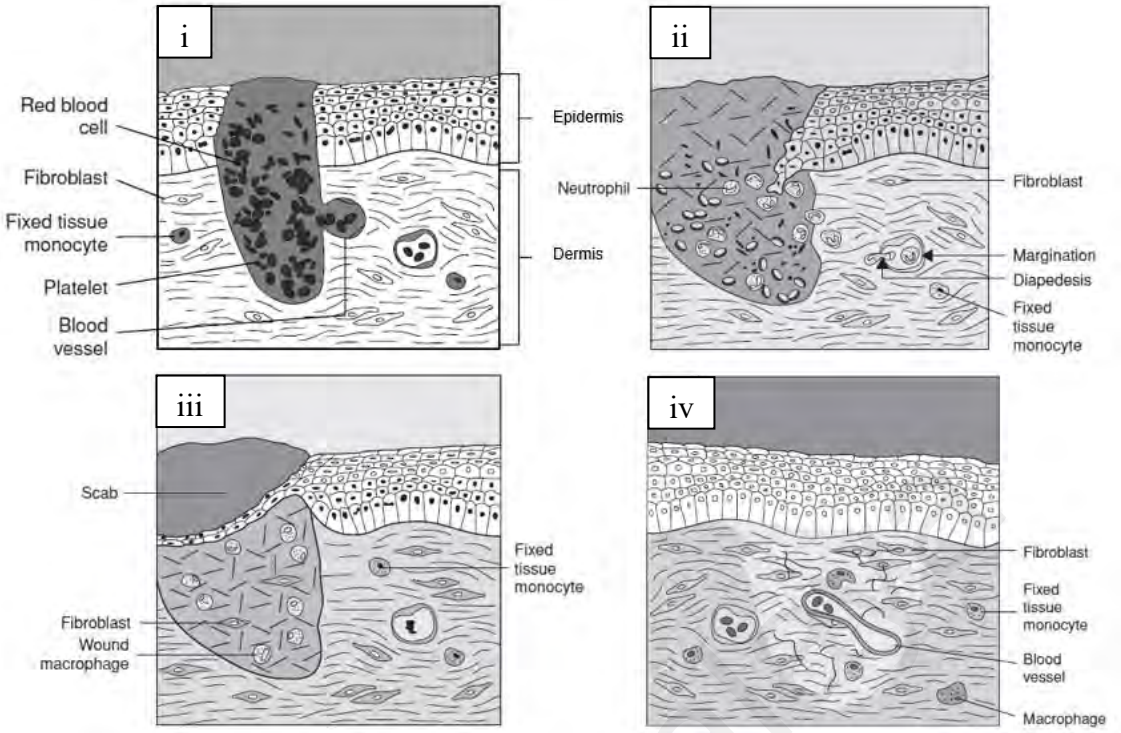

Figure 2.5: Phases in wound healing (Schultz *et al.*, 2011).

2.3 Factors Affecting the Wound Healing Process

There are many factors that influence the wound healing process. These factors can be divided into two main categories, which are intrinstic and extrinsic factors. Despite categorized separately, many of these factors are related and show dependency on one another throughout the wound healing process.

While wound healing is a continuous and ongoing process, there are issues circulating in relation to its effectiveness over time. Firstly, are not all wounds showing healing challenges are similar. Secondly, no two patients have the ability to heal and potential of recovery at the same time. Thirdly, the healing of wound might be affected according to the patients' intentions and desires while recovering. Fourthly, not all wounds are able to heal at a similar rate to other wounds. The fifth issue is healing requires time and patience as it undergoes a series of phases to fully recover. Sixth, different type of wounds requires specific treatments that can provide the maximum healing outcome.

The final issue is that the determination of proximate prevention measures to ensure total wound healing is already defined. However, the stubbornness of patients' in neglecting these measures result in a recurring wound and longer time to fully heal (Strauss *et al.*, n.d.).

2.3.1 Intrinsic Factors

Intrinsic factors are internally-caused factors that affects the performance of bodily functions (Cutting, 1994) and also factors which are related to the overall health of an individual together with any predisposed factors that can affect definite wound healing (Gantwerker & Hom, 2012).

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Age

Wounds occurring in older individuals tend to heal at a slower rate than those in younger individuals. This can be an effect from one or a few co-morbidities such as diabetes and obesity which may occur within an individual as they age over time. In relation to age, skin components tend to show reduced elasticity, reduced thickness of the dermis and diminished capacity of keratinocyte proliferation and migration towards the epidermis which can increase the impact of mechanical stress on the skin (Gould & Fulton, 2016). This also includes poor nutritional intake, uneven hormonal responses within the body, lack of hydration, a range of compromised body systems which is capable to delay wound healing within elder individuals (Gantwerker & Hom, 2012). In addition, elderly individuals have a thinner epidermal layer of the skin, slower cell migratory and slower inflammatory and proliferation responses within wounds. They are also prone to develop chronic diseases at which when

combined with wound occurrence, these individuals would have a more slower wound healing rate and are more exposed to develop wound problems (Singh *et al.*, 2017).

Nutrition

Exhaustion of micronutrients has a direct involvement in the immune system and subsequently, wound healing. While glucose is the main power source for cells to initiate wound healing, malnutrition involving protein and amino acids deficiency are involved with delayed wound healing due to extended periods of inflammation, slower rate of cell proliferation and delayed angiogenesis. Besides, micronutrients such as vitamins and minerals play an important part within the immune system of the body and in wound healing itself.

Furthermore, vitamin C deficiency is related to wound healing, by means of its involvement in modifying collagen (Broughton *et al.*, 2006). Numerous essential nutrients are needed for normal wound healing to properly occur. This includes vitamin A, carbohydrates and omega-3 fatty acids, which are essential for epidermal growth, synthesis of collagen and for regulation of fatty acid pathways (Singh *et al.,* 2017). Therefore, any depletion of crucial macronutrients and micronutrients may give an adverse effect towards the main phases involved wound healing and delay the wound healing process indefinitely.

Disease

Certain diseases such as diabetes in individuals can result in a poor healing effect on their wounds. Wound healing depends on the balance of vessel growth together with proliferation of vessel maturation. However, diabetes can severely affect this balance and interrupt wound healing, tissue regeneration and interfere with the reinstation process of an up-and-running vascular system upon wound incision. This can be shown through delayed angiogenesis in wound healing of diabetic individuals (Okonkwo & Dipietro, 2017). Subsequently, insulin plays a major part in glucose metabolism, synthesis of protein, proliferation and differentiation of multiple cells and the synthesized growth factors which is able to affect the important phases in wound healing such as inflammation and proliferation stages (Apikoglu-Rabus *et al.*, 2009). A diet consisting of excessive carbohydrates and fats instead of proteins, vitamins and minerals adversely put obese individuals with increased possibility of malnutrition (Gould & Fulton, 2016). Therefore, diseases incurred within individuals, especially elder individuals can easily delay the wound healing process.

Psychophysiological Stress

Psychological stress can give a significant negative impact on an individual's health state and may incite behavioural issues. A study carried out by Cole-King & Harding (2001) showed patients with hospital anxiety and depression (HAD) had a significantly lower wound healing rate. Not only that, but stress can also be linked to many diseases including cancer, diabetes and slowed wound healing. Previous studies involving both human and animal models showed significant delays in wound healing. Stress regulates glucocorticoids (GCs) and decreases growth hormones such as IL-1,

IL-6 and TNF-α at the wound site, slowing down inflammation phase. Furthermore, stress brings about emotional distress including depression and anxiety which can reflect on an individual's overall health (Guo & Dipietro, 2010). Figure 2.6 shows the effects of stress and the mediators that brings about compromised and delayed wound healing.

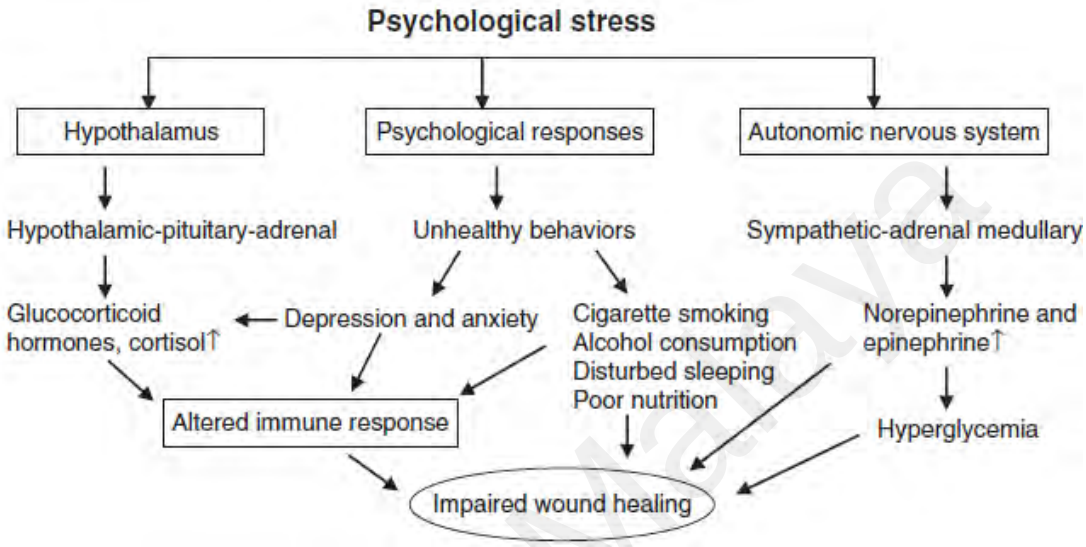

Figure 2.6: Effects and causes of wound healing. Stress-induced and compromised healing of wounds are realized through the hypothalamus, autonomic nervous system and psychological responses of an individual. These causes and effects allow negative responses to flourish and reduce the inflammatory and proliferative growth hormones to impede the wound healing process (Guo & Dipietro, 2010).

2.3.2 Extrinsic Factors

Extrinsic factors are environmental (external) factors surrounding the wound which can effect directly on the wound healing by shortening or prolonging the healing duration of a wound (Gantwerker & Hom, 2012).

Infection

Infections are mainly caused by bacterial presence within the wounds. Bacterial amounts surpassing 10^5 per gram of tissue can cause infection and slow down the healing

process (Gantwerker & Hom, 2012). Bacterial infection on the wound and foreign bodies impede healing by extending the inflammatory phase, reduce wound contraction and collagen release (Broughton *et al.*, 2006). Upon skin injury, microorganisms on the skin surface would be able to gain access to the exposed tissue beneath the skin. In bacterial infection of the skin, infections can be classified as either bacterial contamination, bacterial colonization, localized bacterial infection or critical bacterial colonization and bacterial spreading invasive infection (Guo & Dipietro, 2010).

Hypoxia

In fresh wounds the amount of oxygen is severely reduced due to its consumption from active cells, making the surrounding environment very hypoxic. In certain conditions where oxygen is not restored, the wound healing process is disturbed. However, short-term hypoxia can induce wound healing by secreting growth factors and cytokines from fibroblasts, keratinocytes and macrophages which in return promote cell proliferation, migration and angiogenesis. ROS such as hydrogen peroxide (H_2O_2), in small amounts can act as cellular messengers to promote wound healing in cell motility, synthesizing cytokines and angiogenesis. Hypoxic conditions can increase ROS production. However, high levels of ROS can induce probable tissue damage and halt the wound healing process altogether (Rodriguez et *al.*, 2008; Guo & Dipietro, 2010).

Physical Stress

Physical stress that is directly applied on the wound can lead to hindered wound healing. The types of physical stress include pressure onto the skin or wound area, friction of the skin and shearing of the skin. Firstly, sufficient pressure can be applied at distinct cuts leading to reduced or stops blood flow to the wound site, leading to hypoxic conditions. Secondly, friction of the skin occurs when the surface of the skin or wound rubs off another surface such as clothing or bed sheets. The friction caused may result in abrasion of the healing site, removing healthy granulated tissue and removing the protective layer during the late phase of wound healing. Shearing that occurs when the surface of the skin or wound is intact while the tissues beneath surrounding the wound site is forced to move at an opposing direction. A healing wound can be impeded by shearing as it can cause impairment of new capillaries and tissue formation (Cutting, 1994).

Medication

According to Guo & Dipietro (2010), as cited by Khalil *et al.* (2017) non-steroidal anti-inflammatory drugs (NSAIDs) and chemotherapeutic drugs are the two types of medication that can increase the wound healing period. However, the administration of chemotherapeutic drugs showed statistically promising results as compared to NSAIDs. Guo & Dipietro (2010) also reported on the differences of chemotherapeutic drugs, NSAIDs and GCs steroids on healing of wounds. In relation to studies carried out by Khalil *et al.* (2017), the study proposed by Guo & Dipietro (2010) showed consistent results and depicting chemotherapeutic drugs slow down the inflammation phase, resulting in an extended period time of wound healing. The results for NSAIDs also

showed consistency in outcomes whereby the drugs consumed can interfere with wound healing. The interference mechanism is done through the halting of the proliferation process and can cause hovering in between the late phases of wound healing without moving on to the final phase. Limited amounts of GCs are able to further improve wound healing. Table 2.4 below shows the few types of common drugs which can slow down the healing process of wounds.

Table 2.4: Common drug types that hinder the wound healing process.

Drugs	**Drug Class**
Aspirin	Non-steroidal anti-inflammatory drug
Capecitabine	Chemotherapeutic
Corticosteroids	Immunosuppressant
Dipyrimadole	Platelet aggregation inhibitor
Ibuprofen	Non-steroidal anti-inflammatory drug
Mycophenolate	Immunosuppressant
Warfarin	Anticoagulant

[Adapted from Levine, (2017)]

Lifestyle

The lifestyle of an individual can affect the outcome of wound healing over an extended period. Such lifestyles include the act of smoking and consumption of alcoholic beverages. A poisonous alkaloid, also known as nicotine can be found in tobacco products. Continuous amounts of nicotine during the wound healing phases can reduce blood flow through vasoconstriction and cuts off the immune response leading to a possibility of an increase in infection. In later stages of healing, consumption of tobacco

products hinders collagen production, loosens scar tissue and leaves a higher possibility of wound formation recurrence.

In wound healing, individuals who drink alcohol on a daily basis have to face reduced inflammatory responses, a decline in migration of fibroblast, delayed angiogenesis, reduced Type I collagen and the formation of weakened scar tissue during the late phases of wound healing (Anderson & Hamm, 2014). Furthermore, clinical data comparing continuous alcohol exposure showed a prevalence to compromised wound healing and shows a higher possibility of infection to localize on the wound area (Guo & Dipietro, 2010).

2.4 Medicinal Plants

Medicinal plants have always been the base and foundation in traditional medicine for more than thousands of years which have been used by the Greeks, Chinese, Indians, Arabs and Pakistanis (Khan, 2014) by providing possible cures for diseases back then when modern medicine was not yet discovered. Medicinal plants offer a reservoir of ingredients, containing resources for the development and production of drugs (Singh, 2015).

There are three aspects what makes a plant can be considered as a medicinal plant. Firstly, it can act synergistically, where all of the ingredients contained within the plant work together to act upon a possibly negative target that can cause damage to the human body or system. Secondly, the plant possesses preventive properties, which make it able to hinder the emergence of certain diseases. The preventive act is through the utilization of the readily available components in the plants that are characterized according to its different abilities. The third aspect includes that the medicinal plants are able to support

the administration in modern medicine effectively such as for the treatment of cancer disease (Rasool Hassan, 2012; Singh, 2015).

Nowadays, numerous medicinal plants from all around the world have been documented together with their medicinal properties and chemical constituents that contribute to the treatment of many illness. Plants used as medicine are known as phytomedicine. Phytomedicine is the utilization of plants as medicine and therapeutic functions in treating diseases. Phytochemicals, or secondary metabolites are active ingredients abundant in plants that aid in plants' defence system against microorganism infections and pest infestations. Properly extracted, these phytochemicals possess therapeutic properties that functions similarly to modern-day drugs (Shakya, 2016).

2.4.1 Selected Plants Used Traditionally for Wound Healing

Numerous plants are used traditionally by different tribes in different countries especially in Asia to treat wounds and burns (Thakur *et al.*, 2011). A common example of plant that is widely used to treat wound is *Aloe vera*. *A. vera* has firstly been used by the Egyptians to relieve wounds, burn wounds and infections before the Greeks, Spanish and Africans applied the *A. vera* extracts for multiple different purposes. According to Garcia-Orue *et al.* (2017), as cited by Shedoeva *et al.* (2019), saponins, anthraquinones, glycosides, oleic acid are the few of the many phytochemicals available upon extraction from *A. vera*.

According to Shakya (2016), *A. vera* has wound healing properties and antiseptic effects. The inner gel of *A. vera* leaves also show antiseptic properties (Mahor & Ali, 2016). *A. vera* provides a solution to treat minor burns, incision wounds and sunburns whereby the juice and aqueous extracts from the leaves contain healing

properties in relation to wound healing. In wound healing, the administration of aloe gel benefits the wounded area by increasing blood flow to injured sites, activates angiogenesis, increases proliferation of fibroblast by exhibiting anti-inflammatory and anti-microbial activities (Hosseinimehr *et al.*, 2010; Singh *et al.*, 2011; Farzadinia *et al.*, 2016).

A study carried out by Fox *et al.* (2017) indicated the utilization of aloe gel instead of whole-leaf parts of *A. vera* showed a better and rapid response to wound healing in *in vitro* model. In addition, the study presented results showing the rate of cell migration was also greater with aloe ingredients present when compared with the control. However, the gel-like substance showed a better migration rate of cells as compared to the whole-leaf of *A. vera*. According to Moriyama *et al.* (2016), numerous previous studies have been done *in vivo* involving animal models and clinical trials.

However, only a few studies have been carried out to determine the positive effects of *A. vera* on keratinocyte proliferation. The study carried out by Moriyama *et al.* (2016) targets the effects of *A. vera* gel on migration, proliferation and differentiation of human primary epidermal keratinocytes (HPEKs) in *in vitro* wound healing. The outcome showed that the *A. vera* gel had a very significant effect in proliferating the keratinocytes within 48 hours of treatment, showing indication of cell proliferation increases leads to a better migration of cells across the denuded area and aids in the closure of the wound.

Centella asiatica, commonly known as pennywort or pegaga among Malaysians, is a type of herb which has been formulated into ointment, cream and gels and used in wound healing treatment (Sabale *et al.*, 2012). *C. asiatica* is effective for the treatment of systemic scleroderma, abnormal scarring and keloids due to the asiaticoside, asiatic acid and madecassic acid contained in its extracts (Pawar & Toppo, 2012). Asiaticoside is one

of the main phytocompound which can be detected in *C. asiatica* upon extraction. This compound aids in the regulation of wound healing activity (Roy & Bharadvaja, 2017). According to Somboonwong *et al.* (2012), *C. asiatica* functions in wound healing by reducing inflammation, induces angiogenesis by exhibiting antioxidant properties at the wound site. It also accelerates cell growth and cell proliferation in injured sites to bring about proper wound healing. A study carried out by Ahmed *et al*. (2019) on wound healing in rabbits using *C. asiatica* hydrogel similar and positive results when compared to a positive control, and healed faster when compared to blank hydrogel treatment and no treatment (negative control).

Furthermore, animals treated with the *C. asiatica* hydrogel showed a reduced wound area when compared to the other three groups. In addition, after five days post-treatment of *C. asiatica*-infused hydrogel, results showed a closed wound and a formation of a thin epidermis above the enclosed wound. Lastly, *C. asiatica* also exhibited wound healing properties in a previous study by accelerating fibroblast proliferation and collagen synthesis, which covers up the wound to a close (Yao *et al.*, 2015; Prakash *et al.*, 2017).

Another common Malaysian plant for wound healing and widely used in food preparation is *Curcuma longa*, or commonly known as turmeric or kunyit in Malay. The main chemical compound present in this Asian spice is curcumin, a yellow phytochemical that is topically administered onto cutaneous wound (Biswas & Mukherjee, 2003; Shah & Amini-Nik, 2017). Curcumin plays an important part in wound healing and has the capacity to do so by increasing tissue granulation, deposition of collagen, tissue remodeling and contraction of the wound to a closure (Akbik *et al.*, 2014; Dan *et al.*, 2018).

Curcumin has anti-inflammatory properties, making wound healing possible. In addition, *in vitro* studies have shown curcumin acts in the regulation of growth factors specifically in suppressing TNF-α and IL-1 production by macrophages (Chan, 1995; Aggarwal *et al.*, 2013). During proliferation phase, curcumin acts by eliminating inflammatory cells from the wound area. In remodeling model, curcumin application can increase growth factor TGF-β, correspondingly increases fibroblast proliferation and promotes wound contraction (Barchitta *et al.*, 2019). Freshly prepared *C. longa* herbal applied topically in albino rats showed fast wound closure and reduced wound area when compared with control groups (Dons & Soosairaj, 2018).

A study carried out by Sharma *et al.* (2018) used human keratinocyte (HaCaT) cells, a combination of curcumin and hyaluronic acid showed better wound healing properties than applied as individual agents. This study supports the antioxidant and anti-inflammatory properties of *C. longa* possess which is imperative in wound healing. Wound closure showed dose-dependent results, whereby higher dosage of curcumin-infused hyaluronic acid showed better cell viability as the concentration increased. Conversely, when only curcumin was used, it resulted in less cell viability, indicating an increment of toxicity towards the cells when administered individually.

2.5 *Tabernaemontana* Species

Tabernaemontana species belongs to the family Apocynaceae. The family Apocynaceae make up 3,700 species in 424 genera (Endress & Bruyns, 2000). It is a tropical and subtropical species that can often be found in Africa, America and Asia (Silveira *et al*., 2017). According to Rumzhum *et al.* (2012), *Tabernaemontana divaricata* (Figure 2.7) is a green plant shrub, with a height of 54 cm has shiny leaves and crepe jasmines as flowers. In tropical countries, it is a garden plant that is commonly used due to its abundance of alkaloids.

In general, *Tabernaemontana* species has long be used in traditional medicine as it possesses cytotoxicity, anthelminthic, analgesic and antinematodal activities (Abubakar & Loh, 2016). Among the Malay ethnic in Sarawak, freshly pounded leaves of *T. divaricata* is traditionally applied to inflamed joints and used to relieve inflammation (Chai, 2006).

Figure 2.7: *Tabernaemontana divaricata* (Rahman & Akter, 2015).

Taxonomical classification of *Tabernemontana divaricata*:

Kingdom : Plantae

Division : Magnoliophyta

Class : Magnoliopsida

Subclass : Asteridae

Order : Gentianales

Family : Apocynaceae

Genus : *Tabernaemontana*

Species : *Tabernaemontana divaricata (*L.)

Information taken from United States Department of Agriculture (USDA), Natural Resource Conservation Service (NRCS) website, retrieved March 12th 2020.

2.5.1 Biological Activities Reported on *T. divaricata* Species

T. divaricata extracts have been investigated to study its cytotoxicity towards cancerous cells. In a study carried out by Thind *et al.* (2008), multiple solvents consisting of chloroform, hexane, methanol and ethyl acetate of *T. divaricata* leaf extracts were subjected on colon cancer cells of HCT-15, HT-29 and 502713, MCF-7 (breast cancer cells) and PC-3 (prostate cancer cells). Studies show that methanol extract exhibited 71% and 76% inhibition in growth on 502713 and HT-29 cells respectively and chloroform extract showed an efficient growth inhibition of 72% towards HCT-15 cells and 71% for both HT-29 and 502713 cancer cell lines.

In another study, petroleum ether extract and ethanolic extract of *T. divaricata* were evaluated for their anticancer properties on HCT-15 and MCF-7 cells. Results

obtained indicated that *T. divaricata* extracts showed good cytotoxicity towards the cancerous cells with exception of the HCT-15 cell line (Doshi *et al.*, 2017). Another separate study using hydroalcoholic extracts of *T. divaricata* showed a positive hindering effect on the growth of HeLa (human cancer cell line) (Dantu *et al.*, 2012).

A previous study shows that methanol extracts of *T. divaricata* exhibited anti-acetylcholinesterase (AChE inhibitor) in both *in vitro* and *in vivo* model. The *in vitro* study was carried out by Ingkaninan *et al.* (2003) showed that 0.1 mg/ml of methanolic extracts of *T. divaricata* was able to suppress more than 90% of acetylcholinesterase (AChE) activity. The study supported the hypotheses stating (i) the use of miniscule amount of *T. divaricata* methanolic extract *in vivo* is able to suppress the activity involving circulating and cortical AChE and butyrylcholinesterase (BuChE) and (ii) improve neuronal activity in the cerebral cortex in rats (Chattipakorn *et al.*, 2007).

A study on the effects of aqueous and ethanolic extracts of *T. divaricata* inducing catatonia in rats by using an anti-psychotic drug (haloperidol) showed a shorter catalepsy period when higher concentration of extracts ranging 50, 100 and 150 mg/kg were administered to the different test groups. While both of the extract is able to hinder cataleptic activity, ethanolic extract of *T. divaricata* gives a more favourable outcome in reducing catalepsy period when compared to the aqueous extract of *T. divaricata* (Raj *et al.*, 2014).

Apart from being an anti-cataleptic inhibitor, *T. divaricata* extracts are also able to act as a central nervous system (CNS) depressant. A study conducted by Faruq *et al.* (2018) showed that *T. divaricata* extracts reduced stress and anxiety in Swiss albino mice through a series of neuropharmalogical tests. A reduction of immobility duration in the mice during the execution of neuropharmalogical tests indicated

antidepressant activity is shown by the methanolic extracts of *T. divaricata* leaves at concentrations of 200 and 400 mg/kg.

In addition, *T. divaricata* extracts also possess antibacterial properties, supported by a study carried out by Sumitha *et al.* (2015) in which various *T. divaricata* flower extracts were used to study antibacterial activity against ocular pathogens. Furthermore, methanolic extracts obtained from stem bark of *T. divaricata* showed a positive antibacterial activity against all tested bacterial pathogens. Numerous antimicrobial studies of *T. divaricata* properties had been investigated due to its ability to combat against diseases of syphilis, leprosy, gonorrhoea, dysentery, diarrhoea, malaria and as an antiparasitic against worms (van Beek *et al.*, 1984; Pratchayasakul *et al.*, 2008).

The leaves of *T. divaricata* are also reported to show antifungal potential against the fungal pathogen *Candida albicans* (Wankhede *et al.*, 2013). In addition, a minimum inhibitory concentration (MIC) of the *T. divaricata* stem extracts was carried out to determine the minimum amount of methanolic extracts to work against different fungal strains (Kumari *et al.*, 2015). Besides, hydroalcoholic and aqeuous extracts of *T. divaricata* leaves were also showed to possess antidiarrheal activity which affects gastrointestinal motility in rats (Raj *et al*., 2013).

A similar study was carried out by Kumari *et al.* (2018) by using methanolic extracts of leaf of *T. divaricata* to treat diarrhoea in rats. Results produced by the administration of methanolic extracts towards the rats indicated the extract reduces wet faeces amount when a higher dose of the extract is given. The study includes the potential of methanolic extract of *T. divaricata* to be valuable as a diuretic agent. When the higher dose (mg/kg) of *T. divaricata* was administered, there was an increase in urine volume as well as increased urine concentrations of Na^+ and K^+ ions. Ethanolic extracts of *T. divaricata* were also tested in a study to examine any antitussive and anti-asthmatic

activity properties. Preliminary results showed ethanolic extract of *T. divaricata* promotes notable cough inhibition when compared with standard in increasing doses of the given extract as compared to the given standard drug of 0.03 g/ml codeine solution. In addition, results showed that the *T. divaricata* extract might have broncho relaxant and bronchoprotective properties on histamine-induced and acetylcholine-induced respiratory stress in guinea pigs with dose-dependent extract outcomes as compared to the standard drug administered of 1 µg/ml aminophylline (Srivastava *et al.*, 2019). A recent *in vivo* study carried out by Anbukkarasi *et al.* (2020) showed extract-only *T. divaricata* and *T. divaricata* extract biosynthesized with silver nanoparticles (AgNPs) was able to impede selenite-induced cataractogenesis in Wistar rats with the latter being more efficacious.

A study carried out by Santhi *et al.* (2018) showed the effective concentration (EC_{50}) value of 80 µg of flavonoid fraction from *T. divaricata* (TdFf) was able to kill Dalton's lymphoma ascites (DLA)/Erlich's lymphoma ascites tumor cells in *in vitro* model. In addition, *in vivo* antioxidative studies have also shown that TdFf is an excellent producer of enzymic and non-enzymic antioxidants in the liver of Swiss albino mice when compared to the standard silymarin used in the study. The administration of TdFf was able to reduce and halted the growth of tumor cells, implying their successful antitumorigenic activities. Another recent *in vitro* antioxidative and cytotoxicity study utilizing protein fraction from *T. divaricata* leaves were also tested on DLA tumor cells. Studies carried out showed *T. divaricata* exhibited excellent antioxidant activities, as the concentration of the extract increased. Both *in vitro* studies have confirmed that *T. divaricata* has the potential to inhibit the proliferation of DLA cells (Srilatha *et al.*, 2021).

CHAPTER 3: METHODOLOGY

3.1 Preparation of *Tabernaemontana* Extracts

3.1.1 Solvents

Analytical grade methanol used for extraction preparation were purchased from Merck, Germany.

3.1.2 *Tabernaemontana* Collection

Leaves of *Tabernaemontana divaricata* were collected from home garden in Port Dickson, Negeri Sembilan in the month of March, 2019. Leaves of *Tabernaemontana divaricata* 'Flore Pleno' variant were collected from Rimba Ilmu Botanic Garden, Universiti Malaya in the month of April, 2019. All collected specimens were identified and authenticated beforehand by a botanist Dr. Sugumaran Manickam and deposited into the same herbarium.

3.1.3 Preparation of *Tabernaemontana* Extracts

3.1.3.1 Aqueous Extraction

Fresh leaves (20 g) of *T. divaricata* and *T. divaricata* 'Flore Pleno' were cleaned using sterilized distilled water to remove any possible contaminants. The leaves were cut into small pieces with measurements of 1 mm^2, before being heated separately at 80 ºC in 500 ml of distilled water for 1 hour. Aqueous solutions obtained were filtered through a filter paper (Whatman). The filtrates obtained were then collected, freeze-dried, stored in specimen bottle and kept in the dark for future use (Krishnan, 2005).

3.1.3.2 Methanol Extraction

Leaves of *T. divaricata* and *T. divaricata* 'Flore Pleno' were oven dried at a temperature between 35 ºC and 40 ºC before being grounded into powder form. The powder obtained were then soaked separately in methanol with a ratio of 1:10 (w/v) at room temperature for 72 hours. The filtrates obtained were then collected and excess solvents were evaporated under reduced pressure until dryness and this was achieved by using a rotary evaporator at 40 ºC, producing a dark greenish methanol extract. The extracts obtained were stored in a specimen bottle and kept at -20 ºC for future use (Azis *et al.*, 2017).

3.1.3.3 Stock Serial Dilution

Aqueous extracts of *Tabernaemontana* were dissolved in sterilized distilled water, filtered and sterilized through a 0.22 µm filter cap (Sartorius, Germany). Methanol extracts of *Tabernaemontana* were dissolved in dimethyl sulfoxide (DMSO) (R&M Chemicals, UK). Stock of the *Tabernaemontana* extracts in aqueous and methanol were further used to prepare various concentrations of the extracts (ranging from 1, 10, 25, 50, 75 and 100 µg/ml) by 10-fold serial dilutions. (Krishnan, 2005; Azis *et al.*, 2017).

3.2 *In vitro* Wound Healing Assay

3.2.1 Chemicals

Dulbecco's Modified Eagle's Medium (DMEM) was purchased from Sigma-Aldrich, USA. Foetal Bovine Serum (FBS) was purchased from iDNA Labs, South America. Accutase was purchased from ESGRO, USA. HEPES was purchased from Sigma-Aldrich, USA. Sodium bicarbonate ($NaHCO_3$), Neutral Red and formaldehyde were purchased from R&M Chemicals, UK. Calcium chloride ($CaCl_2$) and acetic acid (Glacial) were both purchased from Systerm Chemicals, Malaysia. Analytical grade 95% ethanol was purchased from Chemical Industries Malaya (CIM), Malaysia.

3.2.2 Cell Culture

Normal Human Dermal Fibroblasts (HDFa) cell line (ATCC Product Catalog Number PCS-201-012) was purchased from the American Type Culture Collection (ATCC) (Virginia, USA). HDFa cells were cultured in DMEM supplemented with 10% (v/v) Foetal Bovine Serum (FBS). The cells were maintained in T25 and T75 culture flasks (Nunc, Denmark) and incubated at 37 ºC and 5% carbon dioxide (CO_2). The cells were grown, subcultured and maintained until it has reached 80–90% confluency and can be further used in experiments.

3.2.3 Toxicity Assessment of HDFa Cells via Neutral Red Uptake (NRU) Assay

The NRU Assay developed by Borenfreund & Puerner (1985) was used to determine the viability of HDFa cells *in vitro* model. HDFa cells were detached from culture flasks using accutase as a detachment solution. The detached cells were then centrifuged at 100 ×g for 5 minutes in a centrifuge (Kubota, 2010). The centrifugation process resulted in a pellet formation at the base of the centrifuge tube. The clear solution of the supernatant was first discarded and followed by addition of DMEM media with 10% FBS into the centrifuge tube and the pellet cells were resuspended. Trypan blue 0.4% (w/v) solution (Sigma-Aldrich, USA) was used to assess cell viability through the dye exclusion test and viable cells were counted using a haemocytometer (Hirschmann Techcolor, Germany).

HDFa cells were seeded into a 96-well plate at a density of 3×10^4 cells/ml in DMEM supplemented with 10% FBS and left to incubate for attachment at 37 ºC and 5% CO_2 for 24 hours. Next, a range of *Tabernaemontana* extracts with concentration of 1, 25, 50, 75 and 100 µg/ml were added into the wells and was left to incubate further for another 48 hours. Wells containing cells without any extract was designated as the negative control.

After 72 hours incubation period, the plate was removed from the incubator and the media was discarded. Prior to the experiment, Neutral Red (NR) media solution was prepared by mixing 50 µg/ml of NR solution with DMEM culture media. NR media was added into each well and left to incubate for 3 hours to allow uptake of the NR dye into the lysosomes of the viable cells.

After 3 hours of incubation, the NR media was discarded and a mixture of calcium chloride and formaldehyde were added to rinse the cells in each well and the excess NR

media was discarded. NR resorb solution, a mixture consisting of 95% ethanol, water and acetic acid (glacial) (50:49:1) was further added into each well to break the membrane of the viable cells. The optical density (OD) of the viable cells was read using a Multiskan Go spectrophotometer microplate reader (Thermo Scientific, USA) at wavelength of 540 nanometers (nm). The experiment was performed in triplicates.

3.2.4 *In vitro* Wound Healing Assay of *Tabernaemontana* Extracts

The *in vitro* wound healing assay was carried out according to Liang *et al*. (2007) with slight modifications. Briefly, 1×10^5 cells/ml HDFa cells were seeded in a 24-well plate in DMEM media supplemented with 10% FBS and incubated at 37 ºC and 5% CO_2 for 24 hours. At the end of the incubation period, the culture media were discarded with DMEM media supplemented with 1% FBS in each well. The plates with cells were incubated for another 24 hours and followed with a small linear scratch made on the cell layer using a sterile pipette tip. The media was then removed and the cells were rinsed with phosphate buffer solution (pH 7.4) to remove cellular debris and remaining media. Fresh DMEM media supplemented with 1% FBS and 25 µg/ml of *Tabernaemontana* extracts were added into each well accordingly. DMEM with 10% FBS was designated as the positive control and DMEM with 1% FBS (without any addition of *Tabernaemontana* extracts) were designated as the negative control.

The distance of HDFa cells migration towards the gap of the artificial wound were observed microscopically at 0, 24 and 48 hours. The images of migrated HDFa cells were captured using a digital camera that is attached to an inverted microscope (Leica DMI 3000 B) and a Leica computer software system (Leica Application Suite, LAS Version 4.0). The experiment was performed in triplicate.

3.2.5 Calculations of Wound Width and Wound Closure

Rate of cell migration was calculated using calculations reported by Grada *et al.* (2017) as follow:

$$R_M = \frac{Wi - Wf}{t}$$

where,

R_M = rate of cell migration (nm/h)

Wi = initial wound width (nm)

Wf = final wound width (nm)

t = duration of migration (hour)

Percentage of wound closure was calculated as shown below:

$$\text{Wound closure (\%)} = \left[\frac{A_{t=0h} - A_{t=\Delta h}}{A_{t=\Delta h}}\right] \times 100\%$$

where,

$A_{t=0h}$ = area of the wound measured immediately after scratching ($t = 0h$)

$A_{t=\Delta h}$ = area of the wound measured h hours after the scratch was performed

3.3 Total Phenolic Content and Antioxidant Properties of *Tabernaemontana* Extracts

3.3.1 Chemicals

Folin-Ciocalteau (F-C) reagent, sodium carbonate (Na_2CO_3), gallic acid ($C_7H_6O_5$), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), ferric chloride hexahydrate ($FeCl_3.6H_2O$), sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$) and butylated hydroxyanisole (BHA) ($C_{11}H_{16}O_2$) were purchased from R&M Chemicals, UK. 2,4,6-tri-(pyridyl)-s-triazine (TPTZ) was purchased from Sigma-Aldrich, Switzerland. Ascorbic acid was purchased from Sigma-Aldrich, China. Hydrochloric acid was purchased from Merck, Germany. 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS), potassium persulfate ($K_2S_2O_8$) and 2,2-diphenyl-1-picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich, Germany.

3.3.2 Total Phenolic Content (TPC)

Total phenolic content (TPC) of *Tabernaemontana* extracts was determined using the F-C reagent method with slight modifications according to Sun *et al.* (2007), as cited by Yusof *et al.* (2018). 10 µl of *Tabernaemontana* extracts were diluted in methanol and distilled water respectively and mixed with 75 µl of fresh 10% F-C reagent. After 10 minutes of incubation, the solution was mixed with 75 µl of 2% Na_2CO_3 (w/v) solution. The solution was incubated again in the dark for 45 minutes at room temperature. Absorbance was read at 765 nm using a microplate reader. A gallic acid curve was plotted as a standard for TPC, which was expressed as gallic acid equivalent (mg GAE/g). The experiment was performed in triplicate.

3.3.3 2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) Assay

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity was carried out according to Yusof *et al.* (2018) with minor modifications. Briefly, 2.6 mM $K_2S_2O_8$ and 7 mM ABTS solutions were prepared and mixed and further incubated in the dark at room temperature for 16 hours prior experiments. The absorbance of the resulting blue-green ABTS radical solution was adjusted to 0.7 ± 0.02 before used. Next, 20 µl of *Tabernaemontana* extracts (concentrations range from 0.5–3.0 mg/ml) were mixed with 200 µl of diluted ABTS solution and incubated at room temperature for 10 minutes. Absorbance was read at 734 nm using a microplate reader. Ascorbic acid was designated as a positive reference. The scavenging ability of *Tabernaemontana* extracts was expressed as the IC_{50} value in mg/ml, which was the concentration at which ABTS radicals were scavenged by 50%. The experiment was performed in triplicate.

3.3.4 2,2-Diphenyl-1-picrylhydrazyl (DPPH) Assay

2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed according to Sulaiman & Ooi (2012), as cited by Yusof *et al.* (2018) with slight modifications. Briefly, 50 µl of each *Tabernaemontana* extract (concentrations range from 0.5–3.0 mg/ml) were diluted in methanol and distilled water respectively The extracts solution was further mixed with 150 µl of 1 mM DPPH solution in methanol in the wells of a 96-well plate. The plate was incubated in the dark for 30 minutes and absorbance was measured at 515 nm. Antioxidant activity was expressed by the IC_{50} in mg/ml. Ascorbic acid was used as a standard reference. The experiment was performed in triplicate.

3.3.5 Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) assay was carried out according to Benzie & Strain, (1996) as cited by Mahmud *et al*. (2019) with minor modifications. FRAP reagent was freshly prepared by mixing 10 ml of 300 mM acetate buffer (pH 3.6), 1 ml of 10 mM TPTZ in 40 mM HCl, 1 ml of 20 mM $FeCl_3.6H_2O$ and distilled water at the ratio of 10:1:1:1.2 (v/v) and the mixture was incubated in a water bath for 30 minutes before used. Next, 10 µl of *Tabernaemontana* extract were diluted in methanol and distilled water respectively and added with 300 µl of FRAP reagent and further incubated for 30 minutes at room temperature in the dark. Absorbance was measured using the plate reader at 593 nm. Ascorbic acid was used as a standard and FRAP activity was expressed in milligrams as ferrous equivalent Fe (II) per gram of dried extract [mg Fe (II)/g]. Butylated hydroxyanisole (BHA) was used as a positive control. The experiment was performed in triplicate.

3.4 Protective Effects of *Tabernaemontana* Extracts against Hydroxyl Radical

3.4.1 Toxicity Assessment of Hydrogen Peroxide (H_2O_2)

Following the method described by Md-Piah *et al.* (2010), the assessment of the toxicity of oxidant solutions of hydrogen peroxide (H_2O_2) towards the viability of HDFa cells was assessed. HDFa cells were cultured at a concentration of 3×10^4 cells/ml in a 96-well plate before the addition of hydrogen peroxide (H_2O_2). H_2O_2 was diluted in sterilized distilled water and 10 µl of the diluted H_2O_2 was added into each well in 96-well plate. HDFa cells without the addition of H_2O_2 was designated as the negative control. The plates were incubated for 24 hours. After incubation, the NRU assay as described in 3.2.3 was used to determine the viability of the cells. H_2O_2 concentrations

which induced 50% cell viability was identified and further used in protective effect assessment.

3.4.2 Determination of Protective Effects

Protective effects of the *Tabernaemontana* extracts towards hydroxyl radicals was carried out according to the protocol described by Murrell *et al.* (1990), with slight modification. HDFa cells were seeded with concentration of 3×10^5 cells/ml in 96-well plates. The cells were maintained in DMEM, supplemented with 10% FBS and incubated at 5% CO_2 at 37 °C for 24 hours. Two separate and different set of experiments were performed: (i) Pre-treatment of cells with *Tabernaemontana* extracts for 24 hours prior exposure to 100 µM of H_2O_2 for 1 hour, and (ii) Post-treatment of cells with *Tabernaemontana* extracts for 24 hours after exposure with 100 µM of H_2O_2 for 1 hour.

Ascorbic acid (100 µg/ml) was used as a reference standard. HDFa cells treated with only H_2O_2 was designated as a negative control. The plates for each treatment were incubated for a total of 25 hours and the NRU assay described in 3.2.3 was used to carry out assessment on the protection effects by the *Tabernaemontana* extracts.

3.5 Nitric Oxide (NO) Stimulation Assay

Murine macrophage RAW 264.7 cells were mechanically scraped out from the culture flask and centrifuged at 100 × g at 4 °C for 10 minutes. 5×10^5 cells/ml of the RAW 264.7 cells were seeded into wells of 96-well plate and incubated in 5% CO_2 at 37 °C for 24 hours. After the incubation, culture media was carefully discarded and followed by the addition of fresh DMEM supplemented with 10% FBS. RAW 264.7 cells

were then stimulated with various concentration of *Tabernaemontana* extracts (25, 50, 100 and 500 µg/ml) and 1 µg/ml of lipopolysaccharide (LPS) obtained from *Escherichia coli* 0111:B4 was used as a positive control. The RAW 264.7 cells were further incubated for another 24 hours. Nitric oxide (NO) production was measured in the culture medium and was detected by the Griess reagent using sodium nitrite as the standard. The Griess reagent (0.1% N-(1-naphtyl) ethylenediamide dihydrochloride, 1% sulphanilamide in 5% phosphoric acid) and an equal volume of cell supernatants were mixed and the absorbance was measured at 540 nm. According to Varma *et al.* (2016), the nitrite concentration was calculated and expressed as µM/ml.

3.6 Statistical Analysis

All of the experiments were carried out in triplicate and the results were designated as mean ± SD. Statistical analyses were performed using One-Way Analysis of Variance (ANOVA) followed by Tukey's HSD-post hoc test using SPSS software (SPSS Statistics, Version 26, IBM, USA). The probability, $p < 0.05$ is considered to be statistically significant.

CHAPTER 4: RESULTS

4.1 Plant Collection and Identification

Two types of plants from the *Tabernaemontana* species were used in this study, *Tabernaemontana divaricata* (TD) (Figure 4.1) and *Tabernaemontana divaricata* 'Flore Pleno' variant (TDF) (Figure 4.2), one of the many variants of the *T. divaricata* species. The voucher numbers for *T. divaricata* and *T. divaricata* 'Flore Pleno' variant are KLU 60086 and KLU 49906 respectively.

Figure 4.1: Leaves and flowers of *T. divaricata*.

Figure 4.2: Leaves and flowers of *T. divaricata* 'Flore Pleno' variant.

The part of plant used in this study were the leaves for both species. Identification and authentication of both species were carried out at Rimba Ilmu Botanical Garden by a botanist Dr. Sugumaran Manickam. Voucher specimens of each species (stems, leaves and flowers) used in this study was also deposited into the same herbarium in Universiti Malaya. Table 4.1 summarized the part of the plants used in the study, voucher number, time of collection and collection site of the plants.

Table 4.1: Selected *Tabernaemontana* species used in this study.

Plant species	Part Used	Voucher Numbers	Time of Collection	Collection Sites
T. divaricata	Leaves	KLU 60086	March 2019	Port Dickson, Negeri Sembilan
T. divaricata 'Flore Pleno'	Leaves	KLU 49906	April 2019	Rimba Ilmu Botanic Garden, Universiti Malaya

4.2 Total Yield of *Tabernaemontana* Extracts

Both dried powder of the leaves of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant were subjected to the extraction process in aqueous and methanol. Table 4.2 shows the total amount of yield obtained from aqueous and methanol extracts for both species. Aqueous extracts of *T. divaricata* (TD-A) produced a yield of 8.5% as compared to *T. divaricata* 'Flore Pleno' variant (TDF-A) at 9.5%. However, methanol extract of *T. divaricata* 'Flore Pleno' variant (TDF-M) produced a significantly higher yield at 33.9% than *T. divaricata* (TD-M) at 13.2%.

Table 4.2: Total yield from methanol and aqueous extracts of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant.

Extraction solvent	Yield (%) of the extract	
	T. divaricata	*T. divaricata* 'Flore Pleno'
Aqueous	8.5	9.5
Methanol	13.2	33.9

4.3 *In Vitro* Wound Healing Assay

4.3.1 Cytotoxicity of *T. divaricata* and *T. divaricata* 'Flore Pleno' on HDFa Cells

The cytotoxicity evaluation of TD-M, TDF-M, TD-A and TDF-A extracts in different concentrations ranging from 1–100 µg/ml was carried out by the Neutral Red Uptake (NRU) assay. Figure 4.3 shows the effects on HDFa cell viability after the addition of *Tabernaemontana* extracts in different concentration. Both aqueous and methanol extracts of TD and TDF did not exert any notable toxicity towards the HDFa cells at concentration of 1, 10 and 25 µg/ml when observed in correlation with the negative control (HDFa cells without addition of extracts).

TD-M, TDF-M, TD-A and TDF-A showed a moderate toxicity towards the HDFa cells as the extract concentration increased at 75 and 100 µg/ml. However, TDF-A did not exert any toxicity towards the HDFa cells even at concentration of 75 and 100 µg/ml. At this concentration, HDFa cells continue to grow and proliferate. In general, it can be concluded that as the concentration of the *Tabernaemontana* extract increased, the viability of HDFa cells decreased. The HDFa cell toxicity from the highest to lowest toxicity is TDF-A, TD-A, TD-M and TDF-M accordingly. Preliminary results obtained from this assessment indicated that 25 µg/ml was the suitable *Tabernaemontana* extract concentration to be applied in the *in vitro* scratch assay towards HDFa cells.

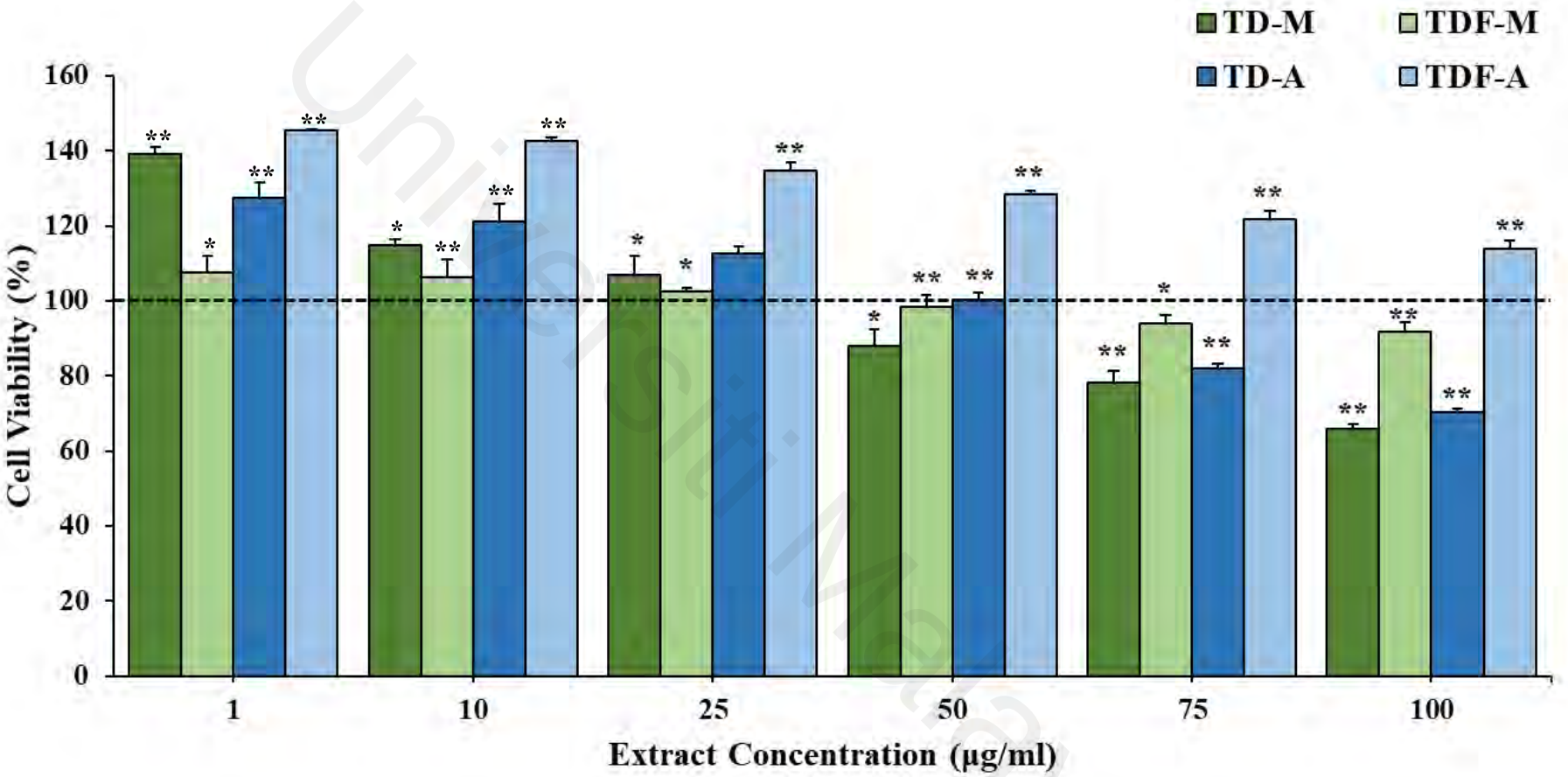

Figure 4.3: Effects of methanol and aqueous extracts of *T. divaricata* (TD-A and TD-M) and *T. divaricata* 'Flore Pleno' variant (TDF-A and TDF-M) on the growth and proliferation of normal human dermal fibroblast (HDFa) cells. Preliminary studies carried out showed aqueous and methanol extracts of both *T. divaricata* and *T. divaricata* 'Flore Pleno' variant did not induce the growth of normal human dermal fibroblast (HDFa) to a certain concentration (1, 10 and 25 µg/ml). The dotted black line indicates the maximum proliferation of HDFa cell prior to the addition of any extracts supplemented with 1% DMEM. Two-way ANOVA followed by Tukey test was used to carry out statistical analysis (*$p < 0.05$ and **$p < 0.001$), (n=3). Note: TD-M, *T. divaricata* methanol extract; TDF-M, *T. divaricata* 'Flore Pleno' variant methanol extract; TD-A, *T. divaricata* aqueous extract; TDF-A; *T. divaricata* 'Flore Pleno' variant aqueous extract.

4.3.2 *In vitro* Wound Healing Assay

HDFa cells were used in this assay to quantify the rate of cell migration and percentage of wound closure within 24 and 48 hours after artificial scratch was made. All the *Tabernaemontana* extracts concentration administered on the HDFa cells were 25 µg/ml upon creation of artificial scratch gap. Figure 4.4 shows images of HDFa cell migration towards the artificial wound area at 0, 24 and 48 hours. The rate of cell migration and percentage of wound closure on HDFa cells were compared with the negative control (cells with DMEM supplemented 1% FBS).

Table 4.3 shows the rate of HDFa cell migration towards the artificial wound gap at 24 and 48 hours after artificial scratch was made. Presence of TD-A and TDF-A in HDFa cell triggered high rate of cell migration at 48 hours, while the rate of migration was lower at 24 hours. In general, presence of aqueous extracts showed highest rate of cell migration within the 24 and 48 hour mark with 4.73 ± 0.17 and 4.18 ± 0.47 nm/h respectively. The rate of HDFa cell migration in descending order is as follows: TD-A > TDF-A > TD-M > TDF-M.

Table 4.4 shows the percentage of wound closure at 24 and 48 hours after artificial scratch was made. Both TD-A and TDF-A showed the highest percentage of HDFa cell migration after 48 hours with migration rate of 58.50 ± 4.30% and 53.54 ± 4.77% respectively. Percentage of wound closure by *Tabernaemontana* in decreasing order is as follows: TD-A > TDF-A > TD-M > TDF-M.

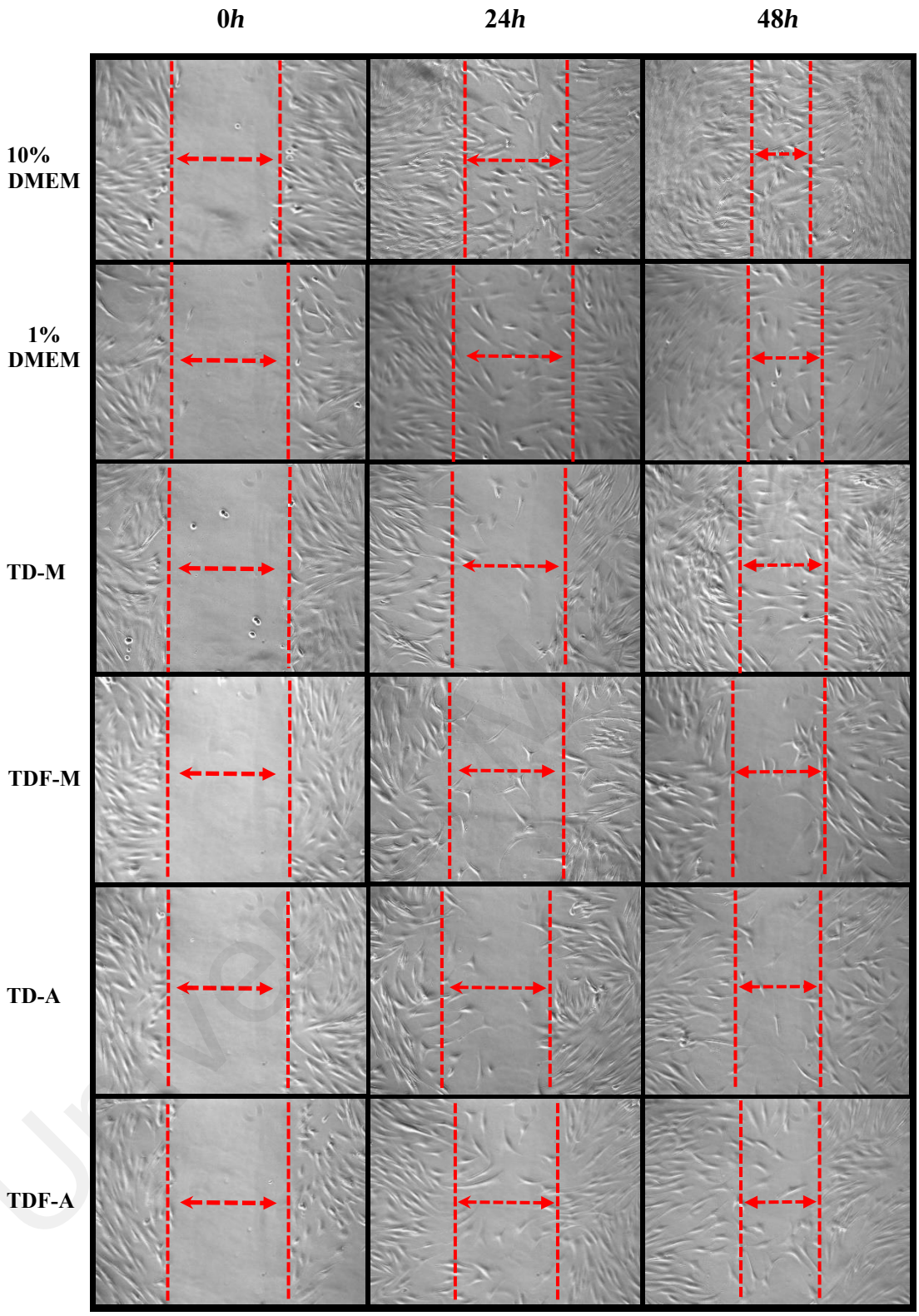

Figure 4.4: Effects of methanol and aqueous *Tabernaemontana* extracts on HDFa cell migration via *in vitro* wound healing model. Images of HDFa cells were taken at an interval of 0, 24 and 48 hours post-treated with 25 µg/ml of TD-M, TDF-M, TD-A and TDF-A. A scale bar of 100 µm was set and images were taken with a 10 × magnification using an inverted microscope. Note: TD-M, *T. divaricata* methanol extract; TDF-M, *T. divaricata* 'Flore Pleno' methanol extract; TD-A, *T. divaricata* aqueous extract; TDF-A, *T. divaricata* 'Flore Pleno' variant aqueous extract.

Table 4.3: Rate of HDFa cell migration (nm/h) was designated as the difference in cell migration from initial to final wound width (nm) over a migration duration (hour, h = 0, 24 and 48).

Extracts	Rate of Cell Migration (nm/h)	
	h = 24	h = 48
10% DMEM	8.51 ± 1.29	5.45 ± 0.47
1% DMEM	8.40 ± 1.29	4.89 ± 0.15
TD-M	4.16 ± 0.23	3.99 ± 0.35^{c}*
TDF-M	4.43 ± 0.32	2.74 ± 0.17^{d}*
TD-A	5.26 ± 4.32	**4.73 ± 0.17^{ab}**
TDF-A	4.89 ± 2.60	**4.18 ± 0.47 ^{ab}**

10% DMEM and 1% DMEM were designated as the positive and negative control respectively. Data above is represented as mean values ± standard deviation (SD). One-way ANOVA was carried out for statistical analysis and mean values ^{abc} in columns indicates there are significant differences at p < 0.05, (n=3). Note: TD-M, *T. divaricata* methanol extract; TDF-M, *T. divaricata* 'Flore Pleno' methanol extract; TD-A, *T. divaricata* aqueous extract; TDF-A, *T. divaricata* 'Flore Pleno' variant aqueous extract.

Table 4.4: Percentage of wound closure (%) was designated as the difference in wound area from initial wound area upon scratch was made ($A_{t=0\mathrm{h}}$) to final wound area, h (hours) = 0 after scratch was performed ($A_{t=\Delta\mathrm{h}}$), Δh = 24 and 48 over the initial area of wound formed upon scratching.

Extracts	Percentage of Wound Closure (%)	
	h = 24	h = 48
10% DMEM	59.64 ± 9.20	90.45 ± 15.27
1% DMEM	44.37 ± 5.48	62.30 ± 2.50
TD-M	13.33 ± 4.53^{ab}*	43.40 ± 4.78^{ab}
TDF-M	4.47 ± 3.95^{b}*	20.87 ± 2.39^{c}
TD-A	21.59 ± 0.96^{ab}*	**58.50 ± 4.30^{a}***
TDF-A	17.24 ± 6.08^{ab}*	**53.54 ± 4.77^{a}***

10% DMEM and 1% DMEM were designated as the positive and negative control respectively. Data above is represented as mean values ± standard deviation (SD). One-way ANOVA was carried out for statistical analysis and mean values ^{abc} in columns indicates there are significant differences at $p < 0.05$, (n=3). Note: TD-M, *T. divaricata* methanol extract; TDF-M, *T. divaricata* 'Flore Pleno' methanol extract; TD-A, *T. divaricata* aqueous extract; TDF-A, *T. divaricata* 'Flore Pleno' variant aqueous extract.

4.4 Total Phenolic Content (TPC) and Antioxidant Activities of *Tabernaemontana* Extracts

4.4.1 Total Phenolic Content (TPC) and Antioxidant Properties

Total phenolic content (TPC) evaluation were carried out on all methanol and aqueous extracts of *Tabernaemontana*. Table 4.5 shows that all the four extracts of *Tabernaemontana* contain high TPC. TPC of both TD-A and TDF-A produced the highest values of TPC with 28.90 ± 1.46 mg GAE/g of dried extract and 31.96 ± 0.39 mg GAE/g of dried extract, respectively.

2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay and 2,2-Diphenyl-1-picrylhydrazyl (DPPH) assay were carried out to determine the antioxidant potential by evaluating the free radical scavenging effect of *Tabernaemontana* extracts. Ferric reducing antioxidant power (FRAP) assay was carried out to determine the reducing potential of *Tabernaemontana* extracts. TDF-A extract showed the highest free radical scavenging activity in ABTS and DPPH assay with IC_{50} values of 1.11 ± 0.03 µg/ml and 2.44 ± 0.02 µg/ml, respectively. Reducing potential of TDF-A extract was also high [18.07 ± 0.03 mM Fe (II)/g] among all the *Tabernaemontana* extracts.

TDF-A extract produced the highest antioxidant activity in all the tested antioxidant assays and the highest TPC value among all the extracts. Inversely, TD-M extract showed the lowest TPC value among all extracts. Radical scavenging activities and reducing potential values are also among the lowest compared to other extracts [IC_{50} = 2.62 ± 0.03 in ABTS assay, IC_{50} = 3.66 ± 0.03 in DPPH assay and 18.94 ± 0.03 mM Fe (II)/g in FRAP assay].

Table 4.5: TPC and antioxidant properties of methanol and aqueous of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant.

Extracts	TPC (mg GAE/g DE)	ABTS IC_{50} (µg/ml)	DPPH IC_{50} (µg/ml)	FRAP (mM Fe (II)/g)
TD-M	14.25 ± 0.15^{d}	2.62 ± 0.03^{b}	3.66 ± 0.03^{a}	18.94 ± 0.02^{a}
TDF-M	19.64 ± 0.14^{c}	2.33 ± 0.09^{a}	3.06 ± 0.01^{b}	17.35 ± 0.04^{d}
TD-A	28.90 ± 1.46^{b}	2.11 ± 0.11^{c}	2.87 ± 0.01^{c}	18.62 ± 0.01^{b}
TDF-A	**31.96 ± 0.39^{a}**	**1.11 ± 0.03^{d}**	**2.44 ± 0.02^{d}**	**18.07 ± 0.03^{c}**

One-way ANOVA was carried out for statistical analysis and mean values ^{abcd} in columns indicates there are significant differences at $p < 0.05$, (n=3). Note: TPC, total phenolic content; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay; DPPH, 2,2-diphenyl-1-picrylhydrazyl assay; FRAP, ferric reducing antioxidant power; TD-M, *T. divaricata* methanol extract; TDF-M, *T. divaricata* 'Flore Pleno' methanol extract; TD-A, *T. divaricata* aqueous extract; TDF-A, *T. divaricata* 'Flore Pleno' aqueous extract.

4.4.2 Correlation between TPC and Antioxidant Activites

Correlation analyses was carried out to determine the relationship between total phenolic content (TPC) and three antioxidant assays (ABTS, DPPH and FRAP) of *Tabernaemontana* extracts. Tabulated results were based on extracts produced the highest TPC value (TD-M) (Table 4.6) and the highest antioxidant values (TDF-A) (Table 4.7).

TD-M extracts showed there were moderate negative correlation between TPC with free radical scavenging potential assays, ABTS (r = -0.509) and DPPH (r = -0.516) respectively. However, there is a moderate positive correlation between TPC with FRAP (r = 0.585). A moderate negative correlation between ABTS with DPPH (r = -0.475) and a significant strong negative correlation between ABTS with FRAP (r = -0.996). Inversely, there is a moderate positive correlation between ABTS with FRAP (r = 0.393).

Table 4.6: Pearson's correlation coefficient (*r*) between total phenolic content (TPC) and antioxidant activites of TD-M extracts.

Parameters	TPC	ABTS	DPPH	FRAP
TPC	1			
ABTS	-0.509	1		
DPPH	-0.516	-0.475	1	
FRAP	0.585	-0.996*	0.393	1

Correlation analysis was carried out for statistical analysis and * indicates there are significant differences at $p < 0.05$. Note: TPC, total phenolic content; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay; DPPH, 2,2-diphenyl-1-picrylhydrazyl assay; FRAP, ferric reducing antioxidant power; TD-M, *T. divaricata* methanol extract.

Correlation analyses of TDF-A extracts showed there was a weak, moderate and strong negative correlation between TPC with ABTS (r = -0.311), DPPH (r = -0.642), and FRAP (r = -0.915) respectively. However, a strong positive correlation was shown among all antioxidant activities between DPPH with ABTS (r = 0.929) and FRAP (r = 0.669), and between DPPH and FRAP (r = 0.897).

Table 4.7: Pearson's correlation coefficient (*r*) between total phenolic content (TPC) and antioxidant activites of TDF-A extracts.

Parameters	TPC	ABTS	DPPH	FRAP
TPC	1			
ABTS	-0.311	1		
DPPH	-0.642	0.929	1	
FRAP	-0.915	0.669	0.897	1

Note: TPC, total phenolic content; ABTS, 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) assay; DPPH, 2,2-diphenyl-1-picrylhydrazyl assay; FRAP, ferric reducing antioxidant power; TD-A, *T. divaricata* 'Flore Pleno' aqueous extract.

4.5 Protective Effects Against Hydroxyl Radical

4.5.1 Toxicity (IC_{50}) Determination of Hydrogen Peroxide (H_2O_2)

The HDFa cells viability after being exposed with hydrogen peroxide (H_2O_2) in varying concentrations (0.001, 0.01, 0.1, 10, 100, 1000, 10000 and 100000 µM) were determined using the Neutral Red Uptake (NRU) assay. Figure 4.5 shows the lowest toxicity of H_2O_2 was recorded at the concentration of 0.001 µM with 94.9% HDFa cell viability. The highest toxicity of H_2O_2 was observed at a concentration of 100000 µM with 24.02% of HDFa cell viability. H_2O_2 addition at a concentration of 100 µM resulted in 53.79% of the cell viability. Therefore, based on these preliminary results H_2O_2 concentration of 100 µM was selected for further application in protective (pre-treatment and post-treatment) studies.

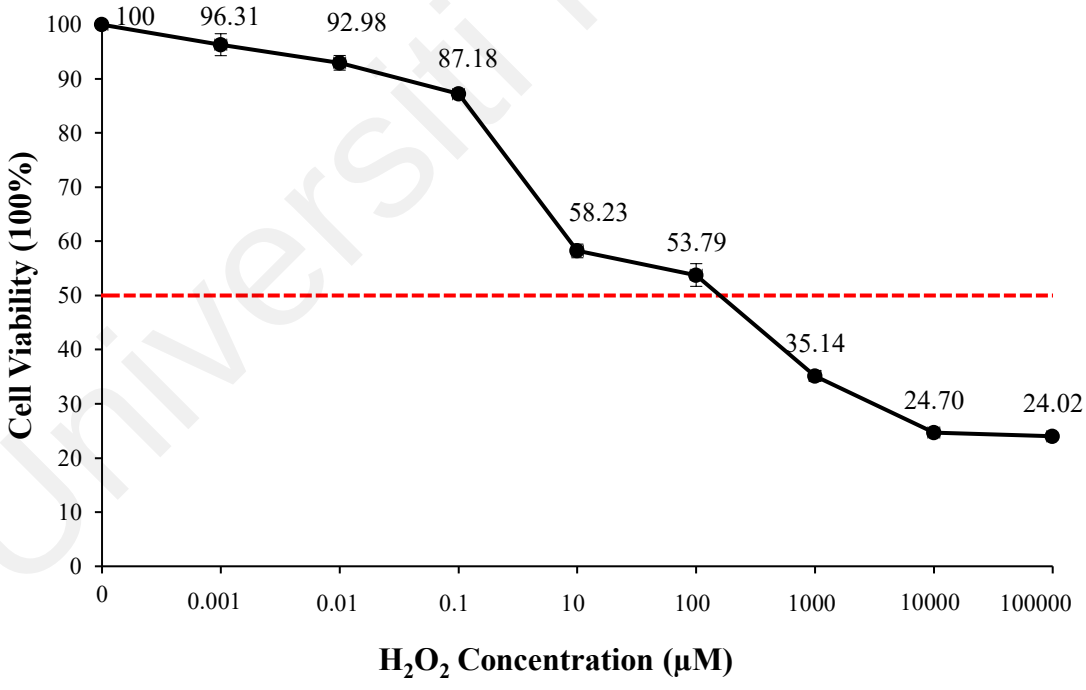

Figure 4.5: HDFa cell viability is determined by hydrogen peroxide (H_2O_2) concentration carried out by the Neutral Red Uptake (NRU) assay. H_2O_2 concentration ranging from 0.001–100000 µM was used to promote toxicity towards the HDFa cells. 50% HDFa cell viability is indicated by the dotted red line.

4.5.2 Determination of Protective Effects

4.5.2.1 Pre-Treatment

For pre-treatment studies, HDFa cells were pre-treated with *Tabernaemontana* extracts (25, 50 and 100 µg/ml) first before being exposed to 100 µM H_2O_2 for 1 hour. As shown in Figure 4.6, pre-treated HDFa cells resulted in a higher protectivity effect by the *Tabernaemontana* extracts. Similar to previous toxicity results of *Tabernaemontana* extracts, protectivity activity towards HDFa cells showed a steady decrease as the extract concentration was increased to 50 and 100 µg/ml. This study showed at 25 µg/ml, extracts TDF-M, TD-M and TDF-A showed a high percentage of cell viability at 93.92 ± 3.05%, 93.09 ± 3.39% and 91.92 ± 0.71%, respectively. However, 25 µg/ml of TD-A showed a much lower percentage in cell viability 78.57 ± 3.40% as compared to the other extracts. All the results obtained were compared with the positive control (100 µg/ml ascorbic acid) at 95.25 ± 0.83% and the negative control (cells treated with 100 µM H_2O_2 only) at 55.41 ± 1.61% carried out in this study.

4.5.2.2 Post-Treatment

For post-treatment studies, HDFa cells were first injured with 100 µM H_2O_2 for 1 hour before adding a series of *Tabernaemontana* extracts (25, 50 and 100 µg/ml). According to Figure 4.7, addition of all the extract in different concentration to HDFa cells resulted in a reduced percentage of cell viability as compared to pre-treatment studies. While the cell viability is lower compared to pre-treatment studies, post-treatment with 25 µg/ml of *Tabernaemontana* extracts towards HDFa cells showed that it was able to induce more than 50% cell viability significantly with both TDF-M (89.69 ± 3.69%) and TD-M (84.59 ± 2.07%), respectively.

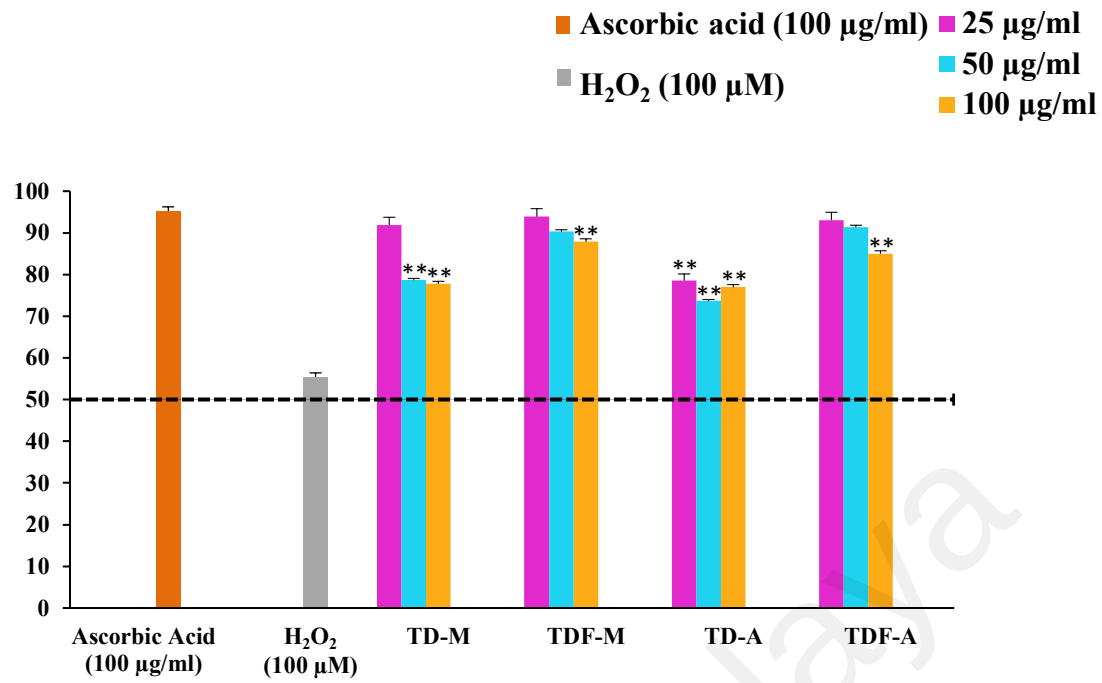

Figure 4.6: Pre-treatment effects of *Tabernaemontana* extracts at 25, 50 and 100 µg/ml towards HDFa cells. HDFa cells were treated with *Tabernaemontana* extracts for 24 hours before exposed to 100 µM H_2O_2 for 1 hour. Results obtained are represented as mean ± standard deviation (SD). Means are significantly different at $*p < 0.05$ and $**p < 0.001$ respectively versus negative control (100µM H_2O_2). 50% HDFa cell viability is indicated by the dotted black line. Note: TD-M, *T. divaricata* methanol extract; TDF-M, *T. divaricata* 'Flore Pleno' methanol extract; TD-A, *T. divaricata* aqueous extract; TDF-A, *T. divaricata* 'Flore Pleno' variant aqueous extract.

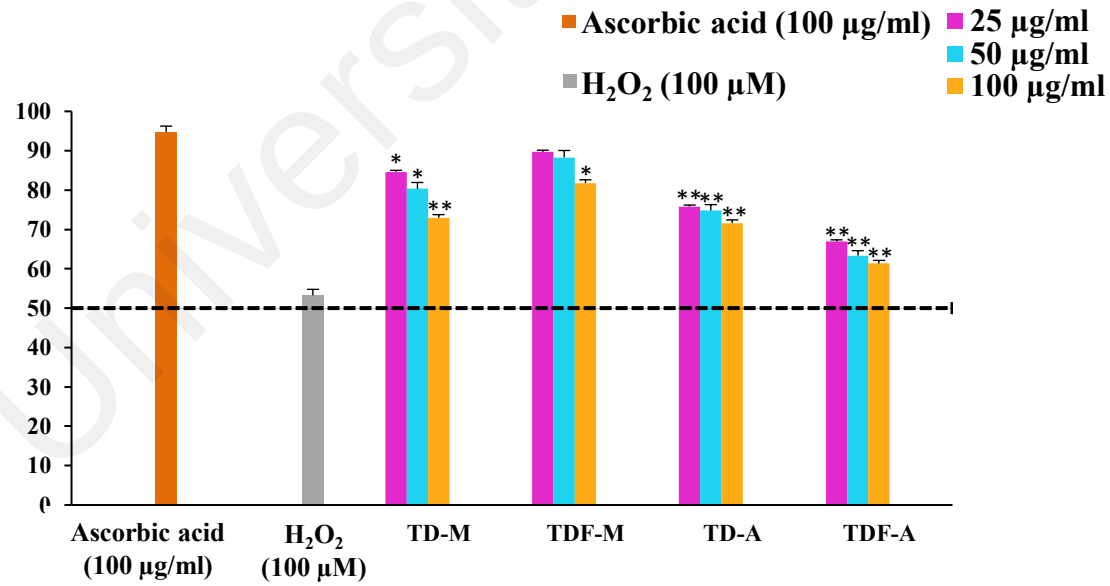

Figure 4.7: Post-treatment effects of *Tabernaemontana* extracts at 25, 50 and 100 µg/ml towards HDFa cells. HDFa cells were exposed to 100 µM H_2O_2 for 1 hour before treated with *Tabernaemontana* extracts for 24 hours. Results obtained are represented as mean ± standard deviation (SD). Means are significantly different at $*p < 0.05$ and $**p < 0.001$ respectively versus negative control (100µM H_2O_2). 50% HDFa cell viability is indicated by the dotted black line. Note: TD-M, *T. divaricata* methanol extract; TDF-M, *T. divaricata* 'Flore Pleno' methanol extract; TD-A, *T. divaricata* aqueous extract; TDF-A, *T. divaricata* 'Flore Pleno' variant aqueous extract.

4.6 Nitric Oxide (NO) Stimulation

Nitric oxide (NO) production was induced from RAW264.7 cells (murine macrophage cells) by exposing the cells to *Tabernaemontana* extracts and lipopolysaccharide (LPS) obtained from *Escherichia coli* 0111:B4. Figure 4.8 shows the amount of NO production stimulated in a dose-dependent (25, 50, 100 and 500 µg/ml) manner by adding methanol and aqueous extracts of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant, accordingly. At 25 and 50 µg/ml, all extracts produced 4.90 to 5.89 µM of NO. At 100 µg/ml of extract concentration, there is a small increase in NO production of 6.26 µM by cells with TD-M and 6.49 µM by cells with TDF-M respectively. Both TD-A and TDF-A were able to inhibit NO production at 500 µg/ml extract concentration, producing 5.22 µM and 5.11 µM of NO respectively. However at 500 µg/ml, NO production increased three times for TD-M (14.66 µM) and TDF-M (14.14 µM). Overall, NO production in increasing order is as follows: TDF-A > TD-A > TDF-M > TD-M.

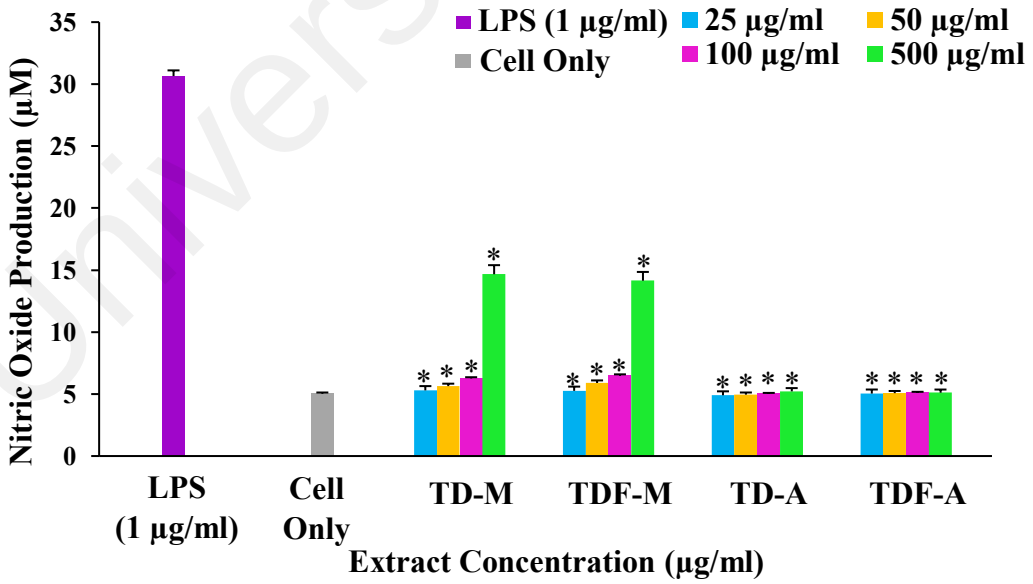

Figure 4. 8: Nitric oxide (NO) production by RAW264.7 cells (murine macrophage cells) were treated with methanol and aqueous extracts of *T. divaricata* and *T. divaricata* 'Flore Pleno' for 24 hours. Results obtained are represented as mean ± standard deviation (SD). Means are significantly different at $*p < 0.05$ versus positive control (1 µg/ml LPS). Note: TD-M, *T. divaricata* methanol extract; TDF-M, *T. divaricata* 'Flore Pleno' methanol extract; TD-A, *T. divaricata* aqueous extract; TDF-A, *T. divaricata* 'Flore Pleno' aqueous extract.

CHAPTER 5: DISCUSSION

5.1 Plant Collection and Identification

Researchers around the globe adhere to the rules of the International Code of Nomenclature for Algae, Fungi and Plants (previously known as International Code of Botanical Nomenclature published in 2011) when identifying plant upon collection and naming one universal and known name for a selected taxon in the classification system. A plant species is documented explicitly by assigning a scientific name according to binomial nomenclature and specifically linked to a botanical specimen or voucher. In addition, authentic and detailed information in regards to taxonomy and binomial nomenclature are imperative for reproducibility and documentation purposes, apart from causing irreparable harm in the science and medical field (Bennett & Balick, 2014). Plant taxonomists and botanists initially depend on visual methods that involve memory and observation skills during species identification. However, they will also depend on botanical specimen that serve as a permanent scientific record which aids in distinguishing the plant species with another species (Bonnet *et al.*, 2018).

A plant's scientific name is uniquely assigned so plants can be identified and classified with the proper information which allows data flow across numerous languages involved in scientific research and various electronic retrieval systems. Furthermore, the use of a common name compared to scientific name of a plant is neither appropriate nor convenient for the scientific community as a plant may be known by multiple names locally or even named so to characterize distinct species with comparable function and taste. A plant is differentiated and placed within a specific taxon from others by means of identification before being assigned a botanical nomenclature. These steps are vital as to allow publication which may establish a prominent plant name that may possibly be

mentioned in the publication abstract or keywords so researchers may have access to the data related to plant in the future (Nesbitt *et al.*, 2010).

Plant classification is also categorized by (i) functional use (medicine, culinary, aromatic and ornamental purposes), (ii) phytochemicals they may contain, (iii) life period and (iv) botanical taxonomy. Botanical taxonomy of medicinal plants is essential in determining its effective medicinal purposes. Taxonomy identification of a selected medical plant would correspondingly establish correct identification of drugs and compounds that may be extracted from the plant. The role of documentation in plant-related research is correlated with the deposited plant voucher into a herbarium for future reference (Alamgir, 2017).

Tabernaemontana divaricata and *Tabernaemontana divaricata* 'Flore Pleno' variant used in this study were collected from their respective collection sites in Malaysia before being identified by a botanist and deposited into the university's herbarium at Rimba Ilmu Botanical Garden located at Universiti Malaya. Voucher numbers were given for both *T. divaricata* and *T. divaricata* 'Flore Pleno' variant before the botanical specimens were deposited into the herbarium.

5.2 Extraction Methods

Preparation of *Tabernaemontana* extracts in this present study was carried out by using both dried samples and fresh leaves of *Tabernaemontana*. For dried samples, leaves were thoroughly cleaned, oven-dried at 35 °C, ground into fine powder and subjected to methanol extraction by maceration process. Aqueous extracts of *Tabernaemontana* were prepared by using fresh leaves immediately upon collection to prevent rotting and deterioration. In general, extract yield obtained from methanol extraction showed a higher yield percentage compared to aqueous extraction from both *Tabernaemontana* species.

In medicinal plant research, fresh and dried plant parts are commonly used for extraction. Fresh leaves of both *Tabernaemontana* species were used for extraction immediately upon collection to preserve sample freshness and prevent any deterioration of phytochemicals that may disintegrate even after a few hours of separation from the main plant after collection. One of the common methods in drying plants is done by oven drying at a given temperature to eliminate moisture entirely. Then, dried plants are ground into powder form and this would increase the surface area between powdered plants and solvents, which subsequently leads to a greater superficial contact with solvents used for extraction. Thus, allowing maximum extraction to occur (Azwanida, 2015). In the present study, both *Tabernaemontana* species were subjected to oven-drying and this designated process applies thermal energy which can retain phytochemicals.

Types of solvent used in extractions depends on the plant, plant part selected to be extracted and the targeted phytochemicals to be extracted. Polar solvents are used in polar compound extraction and non-polar solvents used in non-polar compounds extraction. High polarity solvents such as methanol that was used for this present study for extraction from both *Tabernaemontana* species have shown high efficiency in extracting antioxidant compounds. Methanol is a well-known high polar and organic solvent involved in phenolic extraction from aromatic plants. (Altemimi *et al.*, 2017; Abubakar & Haque, 2020). However, the use of water as a solvent for extraction from both *Tabernaemontana* species has been shown to be effective in phenolic acid extraction with the respective glycosides. It is also able to produce a greater extraction yield than organic solvents such as methanol especially when extracted ultrasonically (Corbin *et al.*, 2015).

The ongoing and expanded research involving the direct application of phytochemicals and naturally-sourced substances extracted from plants is moving to a

more complicated yet sophisticated and modern approach in wound healing treatments due to its readily complex nature involved in physiology and pathophysiology (Sivamani *et al.*, 2012). While naturally-sourced substances obtained especially from plant sources like *Tabernaemontana* species may exhibit numerous beneficial properties, they might not be free from exhibiting side effects as well if improper cleaning and sterilization of plant sources is not been carried out. In this study, the fresh leaves of both *Tabernaemontana* species were rinsed under tap water to ensure removal of any foreign particles or debris which the presence of it in the extracts might lead to possible contamination.

5.3 Wound Healing Activity of *Tabernaemontana* Extracts

In biological screening, *in vitro* experiments are carried out first to determine the therapeutic and biological potential of plant extract before proceeding with *in vivo* models which involve live animals (Stamm *et al.*, 2016). *In vitro* wound healing assessment using scratch assays that was conducted in this study was used to assess the rate of fibroblast cell migration in artificial wound closure. In this scratch assay, a mechanical excision was done to create a cell-free area in a confluent monolayer culture. The induction of cell migration is caused by the exposure of the cell-free area upon damage incurred. The most common way to incise a wound *in vitro* is to scratch the surface by using a pipette tip (Jonkman *et al*., 2014). The wound forms an artificial gap, allowing cell migration to occur over time after the addition of *Tabernaemontana* extract, where the gap is observed over several hours in between through live cell imaging.

Scratch assay was selected in this study as it is reported to be a non-expensive, favourable and common assay conducted to imitate cell migration in wound healing in

in vivo model process (Liang *et al.*, 2007). It was concluded that the two specific applications in the wound healing assays are (i) to determine the quantitative and qualitative analysis of the cell migration under different conditions, and (ii) to study the effects of cell-matrix and cell-cell interactions involved in cell migration (Grada *et al.*, 2017). In the present study, the fibroblast cells undergo proliferation and migrate into the cell-free or wounded area after addition of *Tabernaemontana* extracts, determining one of the event in *in vitro* wound healing process. The use of metrics is also applied for cell migration quantification to determine the wound width and wound area migrated cells. The extent of wound healing was determined by the distance traversed by fibroblast cells migrating into the denuded area.

Fibroblasts play an important role in wound healing as it is directly involved in major processes such as breaking down fibrin clot, produce new extracellular matrix (ECM) and collagen to establish a strong foundation for other cells involved with constructive wound healing and finally contracting the wound to a close (Bainbridge, 2013). Furthermore, fibroblasts also play a critical role in all wound healing phases by removing the ECM, initiates contraction of the wound and new ECM remodeling in the inflammation stage, proliferation stage and remodeling stage respectively (Desjardins-Park *et al.*, 2018). Hence, these are the reasons why fibroblasts were used in this wound healing bioassay. In this present study, extracts of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant shows potential to enhance the wound closure at 48 hours post scratch through fibroblast migration. The possible mechanism might be that the addition of the *T. divaricata* and *T. divaricata* 'Flore Pleno' variant extract, triggered the fibroblasts migration to the wound area to form a scar.

Studies have also shown that during this fibroblast migration, keratinocytes will migrate to the wound surface. Platelet-derived growth factors (PDGFs) also contribute to

the stimulation of fibroblast proliferation and hasten wound closure (Desjardins-Park *et al.*, 2018). Factors that contribute in directing fibroblasts to differentiate into myofibroblasts which promote wound contraction are the signalling molecules from platelets, endothelial cells and macrophages together with transforming growth factor (TGF-β) and PDGF signals (Wilkinson & Hardman, 2020). In the inflammatory phase, interleukin (IL)-6 is a mediator involved in keratinocytes and fibroblasts differentiation, activation and proliferation. Targeting the potential of *T. divaricata* or *T. divaricata* 'Flore Pleno' variant extract in triggering the fibroblast migration is a good strategy in looking for wound healing agent as during wound healing process. Proliferation of wound is initiated with the arrival of fibroblasts which subsequently leads to the formation of a scaffolding consisting of type-I collagen. Final steps in wound healing is the remodeling where a solid scar will be formed, covering the wound to a close. Over a period of months to years, fibroblasts will replace the type-III collagen with type-I collagen to enhance scar flexibility (Johnson *et al.*, 2020).

Alkaloids, tannins, flavonoids and terpenes are some of the natural compounds derived from phytochemicals which are widely reported to promote wound healing (Kumar *et al.*, 2007; Rex *et al.*, 2018). Antioxidant activities, anti-inflammatory and anti-microbial effects are few of the phytochemical-mediated mechanisms involved in accelerating the wound healing process (Shah & Amini-Nik, 2017). Even though components in phytochemicals play a large role in free radical scavenging, warding off infections and wound healing, the identification and formulation of phytochemicals is required to be carried out once it is identified as a possible wound healing agent. Intricate processes such as standardization, safety determination and prior scientific assessment are carried out before the substances can be safely endorsed and prescribed for medicinal purposes (Rex *et al.*, 2018).

Numerous phytochemical studies on *Tabernaemontana* species carried out in previous studies shows that the isolated chemical compounds from *Tabernaemontana* species are related to the biological activity reported in this present study, which includes antioxidant, anti-inflammatory and wound healing. Phytochemical analysis carried out on extracts from latex of *T. divaricata* showed the presence of alkaloids, saponins, flavonoids, tannins, terpenoids and phenolic compounds (Rani & Amena, 2020), which may play a major role and contribute to the overall process of wound healing. Isolation of chemical constituents from *T. divaricata* (reported in this article with the synonym *Ervatamia coronaria*) reported a new bisinadole alkaloid 19,20-dihydroervahanine A and common alkaloids namely coronaridine, heyneanine, voacristine, voacamine and descarbomethoxyvoacamine. Five types of phenolic acids were also identified which are vanillic, gentisic, syringic, 4-hydroxybenzoic and salicylic acid. *T. divaricata* phytochemical isolation and identification showed significant amount of alkaloids with voacristine as its major alkaloidal constituent (Henriques *et al.*, 1996). Van Beek *et al.* (1984) has classified alkaloids of *T. divaricata* into 11 specific classes: vincosan, corynanthean, vallesiachotaman, strychnan, apidospermatan, plumeran, eburan, ibogan, tacaman, bis-indole and miscellaneous. Some non-alkaloidal constituents were also had been reported to be successfully extracted from *T. divaricata* such as terpenoids, steroids, enzymes and hydrocarbons. Rastogi *et al.* (1980) reported eight non-alkaloid constituents from root bark of *T. divaricata* such as α -amyrin acetate, lupeol acetate, α-amyrin lupeol, cycloartenol, β-sitosterol, campesterol, benzoic acid and aurantiamide acetate. The α-amyrin acetate reported to show anti-inflammatory properties while α-amyryl octadecenoate and taraxasterol acetate reported to exhibit possible antioxidant properties, by which all of the stated properties are vital in the continuous process of wound healing (Islam & Lucky, 2019). Bioactivity of certain compounds identified from ethanol extracts from leaves of *T. divaricata* such as

3,7,11,15-tetramethyl-2-hexadecen-1-ol, n-hexadecanoic acid, phytol, squalene, cedrol and vitamin E also reported antioxidant and anti-inflammatory properties (Kalaimagal & Umamaheswari, 2015a). All these reported chemical constituents are mainly terpenoids and phenolic acids, the secondary plant metabolites which had been widely reported to exhibit *in vitro* antioxidant and anti-inflammatory properties (Flores-Sanchez *et al.*, 2002) and related with wound healing activity.

Tabernaemontana species are traditionally used to treat pain, acts as an anti-inflammatory agent and as a topical applicant for treating the wounds (Pratchayasakul *et al*., 2008; Singh *et al.*, 2015). Latex derived from different *Tabernaemontana* species is commonly used topically to treat wounds (Marinho *et al.*, 2016; Naidoo *et al.*, 2021). Readily available proteases from latices in plants functions primarily as a defence mechanism against pests or insects. However, latices are also medicinally important for wound management to stop bleeding and promote wound healing (Urs *et al.*, 2017), as depicted by the *Tabernaemontana* extracts which have protease content from latex utilized in this *in vitro* wound healing study that may correlate with its wound healing properties. Proteases are largely involved in all of the major wound healing physiological processes (Turk, 2006; Craik *et al.*, 2011), and certain identified proteases such as papain (present in Apocynaceae, specifically *E. coronaria*), chymopapain and ficin are topically applied to treat wounds (González-Rábade *et al.*, 2011). Traces of papain content in *T. divaricata* may have contributed to the wound healing outcome observed in this present study. In a recent study carried out by Raju & Rao (2020), phytochemical analysis showed the presence of phenols, alkaloids, flavonoids, amino acids and proteins in the latex of *T. divaricata*. Antioxidant activity and high protease activity of 297.47 µg/ml/min of *T. divaricata* latex

were also reported. These findings are consistent with previously reported results by Rao & Raju (2016).

In a recent study carried out by Santhi *et al.* (2021), it was concluded the purified cysteine protease extracted from latex of *T. divaricata* shows that *T. divaricata* is a potential source of novel protease which can accelerate the wound healing process. Protease also removes damaged tissues as a result of wound formation, and improves wound site from debridement and speeding up the wound healing process (Raju & Rao, 2021). Furthermore, a previous investigation on *T. divaricata* protease reported that it has identified serine protease, which is important for both clot inducing and breaking down clots (Banu *et al.*, 2017). In addition, a recent study carried out by Singh *et al.* (2020) investigated the wound healing abilities (fibrinogenolysis and/or fibrinolysis) of crude and partially purified enzyme (PPE) by measuring the proteolytic activities from both *T. divaricata* latex (115.8 ± 0.3 U/ml) and stem (28.78 ± 0.2 U/ml) as proteases play a major role in wound healing events. All these reports correlate with the present findings on *in vitro* wound healing properties of *T. divaricata* and its ethnomedicinal claim and used as a topical agent in treating wounds or cuts.

5.4 Total Phenolic Content (TPC), Antioxidant Activity and Protective Effect of *Tabernaemontana* Extracts

Phenolic compounds are able to prevent oxidative damage in an array of biomolecules caused by free radicals (Vuolo *et al.*, 2018). Phenolic compounds extracted from different plant parts play an important role in negating with radical species present by means of electron transfer. Phenolic compounds is a type of antioxidant which also acts as an antioxidant defence within the body or cells by preventing the accumulation

and continuous formation of free radicals (Kaurinovic & Vastag, 2019). Antioxidant properties of phenolic compounds are correlated with the efficiency in inactivating ROS by neutralizing the radical completely (Olszowy, 2019). Antioxidants are involved in wound healing by scavenging or reducing any possible free radicals which can cause oxidative stress and further impede or delay the healing process. Upon skin injury, free radicals are produced in wound healing to reduce wound infection and promote skin surface repair. While free radicals act as secondary messengers for immune cells and non-lymphoid cells at the wound site to promote efficacious tissue repair, stimulate angiogenesis and reduce bacterial contamination, excessive amounts of free radical production can damage surrounding epithelial cells, prolong the wound inflammation and hinder the wound healing process (Baron *et al.*, 2020).

In this study, aqueous extracts of both *T. divaricata* and *T. divaricata* 'Flore Pleno' variant in this study showed higher phenolic content but much lower antioxidant values compared to the methanol extracts. However, regardless of type of solvent used, *T. divaricata* produced lower phenolic content values as compared to *T. divaricata* 'Flore Pleno' variant. Conversely to phenolic content values, radical scavenging activities (ABTS and DPPH) and reducing potential activities (FRAP) for *T. divaricata* produced the highest scavenging and reducing potential when compared to *T. divaricata* 'Flore Pleno' variant. Low free radical scavenging activities of *T. divaricata* correlate with high antioxidant activities of the extract. However, high reducing power of *T. divaricata* extracts is correlated with the ability of high number of antioxidants present in extracts to reduce ferric iron (Fe^{3+}) to ferrous iron (Fe^{2+}).

Similar studies on phenolic content and antioxidant assessment of *T. divaricata* had been reported elsewhere. A study by Kalaimagal & Umamaheswari (2015b) showed phenolic content of ethanol extract from flowers, leaves and stem of *T. divaricata* were

6.2 mg GAE/g, 47.1 mg GAE/g and 5.4 mg GAE/g, respectively. The amount of phenolic content most abundant is in the extract from leaves. Another study on phenolic content of aqueous extracts of *T. divaricata* reported 36 mg GAE/g and DPPH scavenging activities of *T. divaricata* showed an IC_{50} = 56.40 mg/ml (Padmaja & Hemalatha, 2011). The phenolic content value mentioned in this previous study is in a good agreement with our current work which produced a range of 14.25–31.96 mg GAE/g values. Methanol extract of *T. divaricata* leaves produced rather low phenolic content values of 0.76 mg GAE/100 g and DPPH radical scavenging activity with an EC_{50} = 417.11 µg/ml (Wasupongpun & Premkaisorn, 2010). Another study on *T. divaricata* leaves reported high phenolic content values of 129 mg GAE/100 g (Srivastava *et al.*, 2013). Phenolic content values obtained from leaves of *T. divaricata* produced 9.68 mg GAE/g of dried weight. DPPH radical scavenging activity of *T. divaricata* was reported at 15.91%. Difference in antioxidant activity reported in the same plant species may be associated with the amount of phenolic compounds that were extracted, the habitat where the *T. divaricata* plants were growing or harvesting time of the plants (Choudhary *et al.*, 2011).

Similarly, there are other reported studies on the phenolic content values and antioxidant activities from other *Tabernaemontana* species. High phenolic content was reported in ethanolic extracts of *Tabernaemontana coronaria* with a value of 98.08 mg GAE/g. DPPH radical scavenging assay and reducing potential of FRAP reported a dose-dependent outcome as the concentrations of extracts used were increased (100–500 µg/ml) (Surya *et al.*, 2011). In addition, ethanol extract of *Tabernaemontana catharinensis* reported very high DPPH radical scavenging activity, IC_{50} = 313.46 ± 0.5 µg/ml when compared to the other fractions obtained by means of column chromatography (Nicola *et al.*, 2013). Methanol extract of *Tabernaemontana*

alterlifolia reported a dose-dependent outcome as the extract concentration increased for ABTS radical scavenging assay (100–600 µg/ml) and DPPH radical scavenging assay (25–250 µg/ml) with each radical scavenging assay able to inhibit 50% of the activity at a concentration of 250 µg/ml and 600 µg/ml respectively (Shrikanth *et al.*, 2015). A study on the antioxidant properties of ethanolic extract from *T. divaricata* leaves showed the extract was able to inhibit DPPH radicals, inhibit NO production, successfully scavenge both anion and free radicals in a dose-dependent manner (200–1000 µg/ml). This study indicates the leaves of *T. divaricata* are abundant in phytochemicals which are able to scavenge free radicals and exhibit antioxidant properties which can be beneficial to human health (Kalaimagal, 2019). In general, the phenolic content and antioxidant activities of the extracts reported from other *Tabernaemontana* species have shown similar findings with the extract of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant.

ROS (reactive oxygen species) is a type of non-reactive free radical in its natural state but it is able to produce free radicals. Such examples of this non-reactive radicals are hypochlorous acid (HClO), ozones (O_3), and hydrogen peroxide (H_2O_2) (Engwa, 2018), and H_2O_2 was used throughout this study. Wound healing process involves the use of ROS in small concentrations to protect against external pathogens and cell signalling for continual survival. An overproduction amount of ROS can induce oxidative damage toward cells, which in return delays healing of recurring wounds (Sanchez *et al.*, 2018). The normal concentration of free radicals in the body is low. Any type of interference which can disrupt and allow a continuous chain reaction of free radicals may result in DNA damage, RNA damage and lipoxygenation, because of oxidative stress. Oxidative stress is depicted as any type of oxygen species disarrangement as a result of reactive oxygen metabolites arrangement imbalance and its protection system by means of antioxidant elimination (Kaurinovic & Vastag, 2019).

In this study, protective effects were carried out by adding methanol and aqueous extracts of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant before (pre-treatment) and after (post-treatment) exposing the HDFa cells to H_2O_2. The focus of this study is to determine the outcome in cell viability based on the concentration and type of which extracts of *Tabernaemontana* is able to provide protectivity towards the cells before and after H_2O_2 exposure.

All the *Tabernaemontana* extracts were able to provide protectivity (indicated by cell viability) towards HDFa cells more than 50%. In general, methanol extracts of *Tabernaemontana* showed the highest protectivity activity in pre-treatment studies. Furthermore, while pre-treatment studies showed a high number of cell viability at the lowest extract concentration, post-treatment application of *Tabernaemontana* extracts can also be considered as an equal treatment for future *in vitro* and *in vivo* studies. Pre-treatment of HDFa cells with *Tabernaemontana* extracts were able to provide an increase in protectivity at a range of 23.16–38.51% while post-treatment of HDFa cells at a range of 13.75–36.43% when compared with the negative control (H_2O_2 only) treated cells at 50% cell viability.

5.5 Nitric Oxide (NO) Production

Nitric oxide (NO) production plays an important role in the wound healing process which comprises of haemostasis, inflammation and antimicrobial action. NO production is also involved in the regulation of cytokines which initiates the inflammatory phase of wound healing (Freedman & Loscalzo, 2003).

At high concentrations in the micromolar (μM) range, NO may exhibit cytotoxic properties as it converts into oxidized intermediates that can cause DNA damage, induce

membrane lipid peroxidation and promotes protein inhibition by means of S-nitrolysation (Burke *et al.*, 2013; Rapozzi *et al.*, 2015). Moreover, the levels of NO also may correlate on the outcome of wound healing. While miniscule amounts of NO may delay wound healing, not enough NO production can disrupt cell migration and lead to definite delays in wound healing. However, if NO production levels are in excess, tissue damage may occur and subsequently impair the ongoing wound healing process. While these are the problems faced, NO production in wound healing can be brought about by endogenous means and exogenous sources (Malone-Povolny *et al.*, 2019) such as plant sources, *T. divaricata* and *T. divaricata* 'Flore Pleno' variant.

As described above, NO is also a free radical which is able to exhibit both protective and cytotoxic effects toward cells (Thomas *et al.*, 2008; Wink *et al.*, 1998). While NO functions as an important mediator molecule involved in multiple and complex physiological reactions, irregular levels of NO can either positively or negatively affect on the outcome of the condition of the human skin (Oliver *et al.*, 2021).

To date, there are very few reported studies done on *in vitro* anti-inflammatory properties of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant. One study shows that ethyl acetate and hexane extracts of *T. divaricata* were able to significantly reduce secretion of pro-inflammatory cytokine TNF-α from cultured RAW264.7 cells by at least 25% (Mueller *et al.*, 2015).

Equally important, there are several *in vivo* studies of *Tabernaemontana* extracts in different solvents which exhibits anti-inflammatory properties. Ethanol extracts of *Tabernaemontana pandaqui* stem was subjected to the anti-inflammatory properties against an induced-inflammatory agent, carrageenin at the back paw (oedema formation) of rats. The outcome of administering 50, 250 and 750 mg/kg of alcoholic extract showed a dose-dependent outcome in inhibiting oedema formation at 3 and 5 hours post

carrageenin-induced inflammation oedema but no effect after 1 hour respectively (Taesotikul *et al.*, 2003). Ethanolic extracts of *Tabernaemontana coronaria* was able to inhibit both acute carrageenin-induced and chronic formalin-induced inflammation rat paw oedema with doses of 100 mg/kg and 250 mg/kg in a dose-dependent manner respectively and can be used in the treatment of high inflammation related disease such as arthritis (Thambi *et al.*, 2006).

Another similar *in vivo* study carried out by Gomes *et al.* (2009) showed ethanol extract of *Tabernaemontana catharinensis* showed inhibitory properties on carrageenan-induced inflammation on rat paw oedema. The highest dosage of *T. catharinensis* at 150 mg/kg showed the highest reduction in oedema reduction after 120 minutes post-carrageenan injection at 42.38%. In addition, another *in vivo* anti-inflammatory study utilizing *T. divaricata* leaf extract on male albino mice was carried out by Jain *et al.* (2013) indicated anti-inflammatory activity is dose-dependabe upon the application of different concentrations of *T. divaricata* leaf extract. Anti-inflammatory activity was observed and depicted by oedema reduction and inhibition after the extracts were applied directly post-induction of inflammation was done on the inner surface of the right ear.

An unrelated *in vitro* study regarding aqueous extracts carried out by Dhanasezhian *et al.* (2018) showed the utilization of aqueous extracts of *Terminalia chebula* fruits with a concentration range from 7.81–250 µg/ml of extracts was able to produce less than 10 µM of NO in total after stimulation of RAW264.7 cells for 24 hours. However, once the concentration was doubled to 500 µg/ml, the NO production by RAW264.7 cells nearly doubled in total which produced more than 10 µM of NO in total.

In general, latest *in vitro* findings showed methanol extracts of both *T. divaricata* and *T. divaricata* 'Flore Pleno' variant extracts were able to produce NO at high extract concentration (500 µg/ml) while aqueous extracts inhibited NO production at high extract concentration. In addition, depending on the medical condition, topical administration of aqueous extracts of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant can be considered in the future as it is non-toxic to cells as compared to other solvents such as methanol while at the same time is able to reduce inflammation phase in wound healing. Moreover, *T. divaricata* and *T. coronaria* has been widely used as an anti-inflammatory agent in Asia but has yet to be studied as an origin for cyclooxygenase (COX) inhibitors which is one of the many important mediators involved in inflammation (Wiart, 2007).

CHAPTER 6: CONCLUSIONS

6.1 Conclusions

To our knowledge, this is the first study carried out to investigate the potential of methanol and aqueous extracts from *Tabernaemontana* species, *Tabernaemontana divaricata* and *Tabernaemontana. divaricata* 'Flore Pleno' variant on wound healing via fibroblast migration in *in vitro* model. Total phenolic content, antioxidant activity via mechanism of free radical scavenging and reducing properties, protective effects and nitric oxide (NO) stimulation were also assessed in this present study. Results obtained showed that aqueous extract of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant are able to provide the highest rate of HDFa cell migration and it shows both extract potential as a wound closure agent. Aqueous extract of *T. divaricata* 'Flore Pleno' variant reported with the highest free radical scavenging and reducing activities with the highest phenolic content among all the extracts. The similar finding was seen in methanol extract of *T. divaricata* where the lowest phenolic content value was reported together with the lowest free radical scavenging and reducing activities. The presence of phenolic content might not be responsible for the observed antioxidant activity in both *Tabernaemontana* species.

In pre- and post-treatment protective effects, all the methanol and aqueous extracts of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant were able to provide protectivity towards HDFa cells against hydrogen peroxide (H_2O_2) which resulted in a high percentage of cell viability after the treatment. Both methanol extract of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant produced NO at high extract concentration while aqueous *T. divaricata* and *T. divaricata* 'Flore Pleno' variant inhibited NO production even at high concentration of extracts. This study shows that methanol and aqueous

extracts of *T. divaricata* and *T. divaricata* 'Flore Pleno' variant were able to produce and inhibit NO production respectively according to the type of extract used.

Before the emergence of modern medicine, the utilization of medicinal plants as a topical applicant has always been an option for the treatment of wound healing. However, while plants may hold the key to unlocking numerous beneficial outcomes, it is best to identify its properties and understand the mechanism and pathways involved which can promote wound healing. Based on the findings, the *Tabernaemontana* species could be considered as a wound healing agent. These findings prove there are evidence backing claims the traditional use of *Tabernaemontana* species in the treatment of wound healing.

6.2 Future Studies

Future studies may include a detailed investigation on the specific phytochemicals from *Tabernaemontana* species which contributes specifically to the mechanism of wound healing. Furthermore, detailed studies on *Tabernaemontana* species which can promote NO production and inhibition in LPS-stimulated RAW264.7 cells should be carried out as this is one of the known ways to screen possible drugs with anti-inflammatory properties.

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