In vitro BIOACTIVITIES OF SINGLE OR COMBINED EXTRACTS FROM THREE SELECTED ASIAN VEGETABLES

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FACULTY OF SCIENCE
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KUALA LUMPUR

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In vitro BIOACTIVITIES OF SINGLE OR COMBINED EXTRACTS FROM THREE SELECTEDTED ASIAN VEGETABLES

ABSTRACT

Three underutilized folklore oriental-based vegetables *Acalypha indica* (AI), *Centella asiatica* (CA), and *Sesbania grandiflora* (SG) have been investigated for pharmacological activities relevant to wound healing process and synergistic actions. Single and combination of aqueous (A) and methanol (M) extracts of the three vegetables namely AI-A, CA-A, SG-A, AI-M, CA-M, SG-M, AI-A+CA-A, AI-A+SG-A, CA-A+SG-A, AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M were assessed for stimulation of wound closure by normal human dermal fibroblast (NHDF) cells, antioxidant, protection against hydrogen peroxide (H_2O_2), stimulation of nitric oxide (NO) from RAW264.7 macrophages and antimicrobial activities. The combined vegetable extracts AI-A+CA-A (90.76%) showed the highest percentage of wound closure, followed by single extract CA-A (89.52%). Pre-treatment of NHDF cells with CA-M at 100 µg/ml offered the best protection against H_2O_2. At 500 µg/mL, CA-A and combination of CA-A+SG-A successfully induced RAW264.7 cells to produce NO of 17.85 µM and 40.84 µM, respectively. All single and combined vegetable extracts showed weak antibacterial properties against gram-negative (*E. coli* and *S. marcescens*) and gram-positive bacteria (*B. cereus*, *B. subtilis*, *M. luteus*, *S. aureus*, and *S. epidermidis*). The single and combined vegetable extract showed antifungal-demelanizing activities against *Aspergillus niger*. AI-A exhibited the highest total phenolic content (TPC) (82.94 mg GAE/g) and moderate reducing activity (61.63 mM Fe (II)/mg) when assessed by ferric reducing antioxidant power (FRAP) assay. Free radical scavenging activity was assessed by 2,2-diphenyl-1-picrylhydrazyl (DPPH) and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay with AI-A+CA-A exhibited good scavenging activity at IC_{50} = 379.75 µg/ml and IC_{50} = 578.7 µg/ml, respectively. Furthermore, identification and

characterization of all the single vegetable extracts via UHPLC (LC-MS/MS) system revealed the major components present might be responsible for all the tested bioactivities. The identified major components were phenolic groups such as simple polyphenols, flavonoids (flavones and flavonols), polysaccharides, and triterpenes (asiaticoside and madecassosides). AI-A+CA-A demonstrated synergism (CI < 1) for migration of fibroblast, DPPH free radical scavenging activity and production of NO. AI-A+SG-A and CA-A+SG-A showed synergism for DPPH free radical scavenging activity and production of NO. AI-M+CA-M and AI-M+SG-M demonstrated synergistic interaction for FRAP reducing activity and production of NO. CA-M+SG-M showed synergism for production of NO. These findings demonstrate the potential formulation by one vegetable or combining extracts from two vegetables with multiple functions of accelerating the migration of fibroblast, an excellent source of antioxidants, antimicrobial, and stimulation of NO production for optimum wound healing properties.

Keywords: *Acalypha indica*, *Centella asiatica*, *Sesbania grandiflora*, synergism, wound healing

BIOAKTIVITI *in vitro* EKSTRAK TUNGGAL ATAU GABUNGAN SAYURAN ASIAN TERPILIH

ABSTRAK

Kajian aktiviti farmakologi berkaitan dengan penyembuhan luka dan tindakan sinergi telah dilakukan ke atas tiga sayuran oriental tradisional yang kurang digunakan *Acalypha indica* (AI), *Centella asiatica* (CA), and *Sesbania grandiflora* (SG). Ekstrak tiga jenis sayuran akueus (A) dan metanol (M) tunggal dan gabungan dinamakan sebagai AI-A, CA-A, SG-A, AI-M, CA-M, SG-M, AI-A+CA-A, AI-A+SG-A, CA-A+SG-A, AI-M+CA-M, AI-M+SG-M, dan CA-M+SG-M dinilai dari segi kebolehan untuk meransang penutupan luka oleh sel fibroblast (NHDF) manusia, antioksida, perlindungan daripada kerosakan saling tindakan oleh hidrogen peroksida (H_2O_2), kebolehan untuk merangsang nitrik oksida (NO) daripada sel makrofaj RAW264.7, dan aktiviti antimikrob. Gabungan AI-A+CA-A (90.76%) menunjukkan peratus penutupan luka yang tertinggi dan diikuti oleh CA-A (89.52%). Pra-rawatan NHDF dengan ekstrak CA-M pada 100 µg/ml memberikan perlindungan paling berkesan terhadap H_2O_2. Pada 500 µg/ml, CA-A dan gabungan CA-A+SG-A berjaya mempengaruhi penghasilan NO oleh sel RAW264.7, dengan masing-masing 17.85 µM dan 40.84 µM. Semua ekstrak tunggal dan gabungan menunjukkan sifat antibakteria yang lemah terhadap spesis bakteria gram-negatif (*E. coli* and *S. marcescens*) dan gram-positif (*B. cereus*, *B. subtilis*, *M. luteus*, *S. aureus*, and *S. epidermidis*). Hanya ekstrak tunggal dan gabungan methanol yang menunjukkan aktiviti antikulat-nyahmelanin terhadap *Aspergillus niger*. AI-A menunjukkan kandungan fenolik tertinggi (82.94 mg GAE/g) dan aktiviti pengurangan oksida yang sederhana (61.63 mM Fe (II)/mg) apabila dinilai oleh asei *ferric reducing antioxidant power* (FRAP). Aktiviti pengaut radikal bebas dinilai oleh asei *2,2-diphenyl-1-picrylhydrazyl* (DPPH) dan *2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)* (ABTS) dan gabungan ekstrak AI-A+CA-A menunjukkan aktiviti terbaik, dengan

masing-masing IC_{50} = 379.75 dan IC_{50} = 578.70 μg/ml. Tambahan lagi, saringan dan pengenalpastian semua ekstrak tunggal dibuat melalui sistem UHPLC (LC-MS/MS) menunjukkan komponen utama yang hadir dan mungkin bertanggungjawab terhadap semua bioaktiviti yang diuji. Komponen utama tersebut adalah kumpulan fenolik seperti polifenol, flavonoid (flavon dan flavonol), polisakarida, dan triterpene (asiaticoside dan madecassoside). AI-A+CA-A menunjukkan sinergism (CI < 1) untuk migrasi fibroblas, pengaut radikal bebas DPPH, dan penghasilan NO. AI-M+CA-M dan AI-M+SG-M menunjukkan interaksi sinergi untuk pengurangan aktiviti FRAP dan penghasilan NO. CA-M+SG-M menunjukkan sinergism untuk penghasilan NO. Penemuan ini menunjukkan potensi formulasi oleh satu sayuran atau gabungan dua sayuran yang mempunyai pelbagai fungsi mempercepatkan migrasi fibroblas, sumber antioksida yang baik, antimikrob, dan stimulasi penghasilan NO untuk penyembuhan luka yang optimum.

Kata kunci: *Acalypha indica*, *Centella asiatica*, *Sesbania grandiflora*, sinergisme, penyembuhan luka

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Norfaizah
Universiti Malaya, 2020

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LIST OF SYMBOLS AND ABBREVIATIONS

-ve	:	Negative
+ve	:	Positive
µl	:	Microliter
µM	:	Micromole
µg/ml	:	Microgram per millilitre
ABTS	:	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
ATCC	:	American Culture Collection
B.C	:	Before Century
cell/ml	:	Cell per millilitre
cfu/ml	:	Colony forming unit per millilitre
CI	:	Combination Index
DMEM	:	Dulbecco's Modified Eagle Medium
DMSO	:	Dimethyl sulfoxide
DNA	:	Deoxyribonucleic acid
DPPH	:	2,2-diphenyl-1-picrylhydrazyl
ECM	:	Extra-cellular matrix
EDTA	:	Ethylene Diamine Tetra Acetic Acid
F-C	:	Follin Ciocalteou reagent
$FeSO_4$	:	Ferrous sulphate
FIC	:	Fractional Inhibitory Concentration
FRAP	:	Ferric Reducing Antioxidant Power
g	:	Gram
GAE	:	Gallic Acid Equivalent
H_2O_2	:	Hydrogen Peroxide

kg	:	Kilogram
kV	:	Kilo volt
L/h	:	Litre per hour
NHDF	:	Normal human dermal fibroblast
NO	:	Nitric Oxide
MBC	:	Minimum Bactericidal Concentration
MDC	:	Minimum Demelanizing Concentration
MFC	:	Minimum Fungicidal Concentration
mg/dl	:	Milligram per decilitre
mg/ml	:	Milligram per millilitre
MIC	:	Minimum Inhibition Concentration
SSI	:	Surgical Site Infection
T	:	Time
U/ml	:	Unit per millilitre
v/v	:	Volume per volume
w/v	:	Weight per volume

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INTRODUCTION

1.1 Research Background

In medical field, wound is one of the major concerns that can be classified into two categories: acute and chronic wound (Singh & McNaught, 2017). Acute wounds heal in a predictable time frame (3–14 days), leads to recovery of anatomical and functional integrity of the wounded area. In contrast, chronic wounds fail to progress through the normal stages of wound healing and takes longer time to heal. Delayed in chronic wounds healing can affect a great number of patients and extremely reduce their quality of life including restricted mobility, loss of employment, and severe patient suffering (Wong et al., 2015; Singh & McNaught, 2017).

In recent years, attention has been turned to the investigation of cost-effective and accessible alternatives for wound therapy. Old-folklore practices or any traditional form of medicine practised for centuries are being studied for their potential in wound care and wound-related disorders. People are dependent more on locally available edible plant materials that offer both nutritional and pharmaceutical (nutraceutical) advantages to humans. In Asian countries, many people consume varieties of nutritionally and medicinally important plants in their daily meals like spinach, coriander, curry leaf, and mint, which are economical and readily available all the time (Raju et al., 2007). Based on the ethnomedicinal report, many vegetables species have a huge potential to be exploited as medicines to treat different ailments, including wound healing.

Today is the era of evidence-based medicine (EBM), scientists are actively screening various plant extracts to discover novel and an effective wound healing drug. Different wound healing models, *in vivo* and *in vitro*, are being developed to verify the traditional claims of wound healing potential from edible plants such as vegetables towards the establishment of risk-free and effective, herbal drugs for wound treatment.

The main key processes in wound healing are the proliferation, migration, and functioning of fibroblasts and keratinocytes cells. The *in vitro* wound healing assays are practical since they are fast, affordable, and can be used to screen a multiplicity of conditions or samples concurrently (Thakur et al., 2011). *In vitro* scratch assay is an easy technique to study the migration of cells, and it mimics to some degree of cell migration in *in vivo* model (Liang et al., 2007).

One of the key aspects in wound healing study is the potential antioxidant of the herbal extracts. Antioxidants can hinder the oxidation process by reacting with free radicals, chelating catalytic metals, and by acting as oxygen scavengers. Excessive amounts of reactive oxygen species (ROSs) such as hydrogen peroxide (H_2O_2) and superoxide (O^2) are considered to be main contributors in delaying the wound healing process (Schafer & Werner, 2008). In addition to ROS, nitric oxide (NO) is a signalling molecule for different pathological events, including inflammatory. In the human immune system, NO probably evolved as a toxic molecule to provide immunity. Another factor affecting wound healing is a microbial infection, such as *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Escherichia coli* infection (Wong et al., 2015). Hence, it is important to look into the antibacterial activity of herbal medicines to eliminate the risk of infections, thus speed the recovery of the wounds.

Malaysia is a country blessed with various sources of medicinal plants like *Eurycoma longifolia* (tongkat ali), *Centella asiatica* (pennywort), *Labisia pumila* (kacip fatimah), *Morinda citrifolia* (mengkudu) and other medicinal herbs. However, we still lack of proper information and standardization of herbal preparation to guarantee their safety, quality, and efficacy (Jantan, 2004). To tackle this problem, adequate research strategy and development need to be established for that our country would be one of the top competitors in pharmaceutical and herbal industries. Therefore, the current study was

carried out to determine the wound healing potential of selected Asian-based vegetables of *Acalypha indica*, *Centella asiatica*, and *Sesbania grandiflora* using *in vitro* model.

1.2 Specific Objectives

The specific objectives of the present study are as follow:

i. to evaluate wound healing effects of the single and combined vegetable extracts of *A. indica*, *C. asiatica*, and *S. grandiflora* on normal human dermal fibroblast (NHDF) cells;

ii. to evaluate antimicrobial effects of the single and combined vegetable extracts of *A. indica*, *C. asiatica*, and *S. grandiflora* on selected bacterial strain and fungus;

iii. to assess the efficacy of the protective effects of the single and combined vegetable extracts of *A. indica*, *C. asiatica*, and *S. grandiflora* against oxidative stress in cellular model; and

iv. to determine the chemical profile of the active vegetable extracts in regard to its wound healing activity.

LITERATURE REVIEW

2.1 Structure and Function of Skin

Skin is the largest organ of the body, takes up roughly 15% of total adult weight and it is made up of three layers: (i) epidermis, (ii) dermis, and (iii) subcutaneous tissue (Figure 2.1) (Kolarsick et al., 2011). It plays vital functions, including as a barrier to separate internal organs of the body and the external environment. In addition, it acts as a shield to protect against foreign agents such as microorganism, heat, radiation, chemicals, and water loss. The epidermis (outer layer) and the dermis (inner layer) are two distinct compartments (Johnstone & Farley, 2005; Evans et al., 2013) separated from each other by a thin layer of extracellular matrix (ECM) proteins called basement membrane (Breitkreutz et al., 2013).

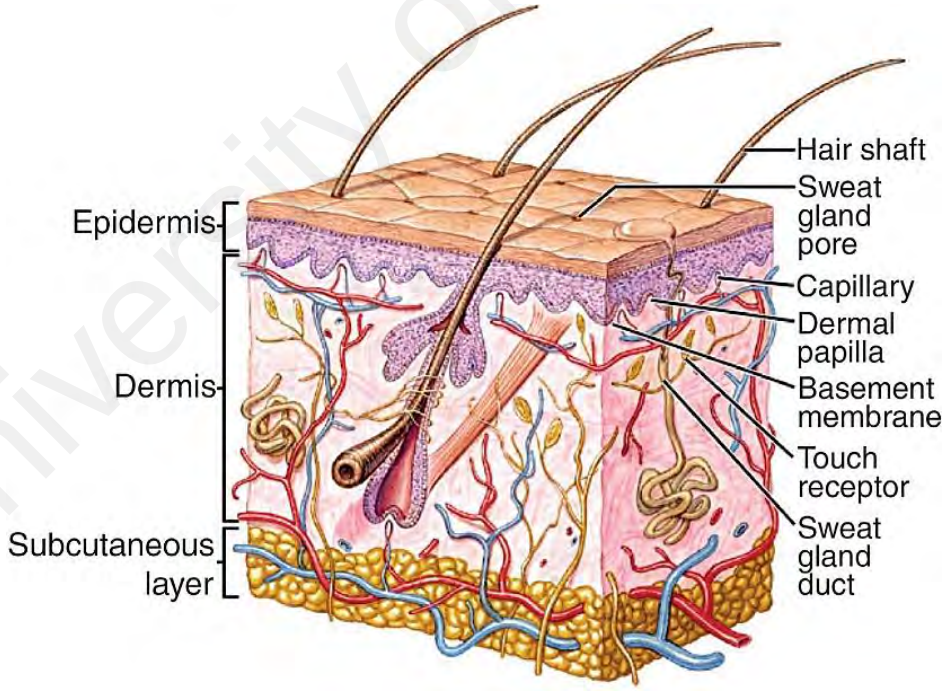


Figure 0.1: Illustration structure of human skin displaying the epidermis (outer layer) and the inner dermal layer (MacNeil, 2008). Reprinted with permission from Elsevier.

The epidermis is made up of stratified squamous epithelium (thin, flat layers of cells attached) organized in four or five layers. This layer is composed of a specific group of cells identified as keratinocytes. The keratinocytes produce keratin, a long, thread-like protein with a protective role (Johnstone & Farley, 2005; Kolarsick et al., 2011). The dermis is a much thicker layer that contains additional accessories such as hair follicles, sweat glands, sensory receptors, and tough connective tissue. It is comprised mainly of ECM proteins such as collagen type I and elastin, fibroblasts, macrophages, and other blood-borne cells (including lymphocytes, plasma cells, and other leukocytes). When the skin is wounded, this layer immediately acts as a mechanical barrier by prevention of infection and thus proceed with a tightly coordinated wound healing process. The subcutaneous layer is the deepest, made up of small lobes of cells known as lipocytes and connective tissue with blood vessels running through it (Kolarsick et al., 2011; Singh & McNaught, 2017).

The skin forms a capable defender against the harmful external environment, sending sensory information and essential in the regulation of body (homeostasis) (Kolarsick et al., 2011). A wound or any breached of the epidermal layer will cause the skin tissue to lose its function. Also, it can cause lesions or illness that reduce the quality of lifestyle and sometimes can even lead to death (Jorge et al., 2008).

2.2 Wound Healing Process

Wounds are the result of skin injuries that interrupt the other soft tissue, and it may be caused by a physical, chemical, thermal, microbial, or immunological insult to the tissue. There are three categories of wound: (i) open wound when the skin is torn, cut, or punctured; ii) closed wound when blunt force trauma causes a contusion; and (iii) burn wounds that are caused by fire, heat, radiation, chemicals, electricity, or sunlight (Shuid et al., 2005; Jalalpure et al., 2008; Jorge et al., 2008).

Wound healing is an orchestrated interaction of cellular and biochemical responses leading to the reconstruction of structural and functional integrity with the recovery of the strength of wounded tissues. It comprises of continuous cell-to-cell interaction and cell-to-matrix interactions. The typical response to injury occurs in three main stages: (i) inflammation, (ii) new tissue formation, and (iii) remodelling (Figure 2.2) (Gurtner et al., 2008; Thakur et al., 2011).

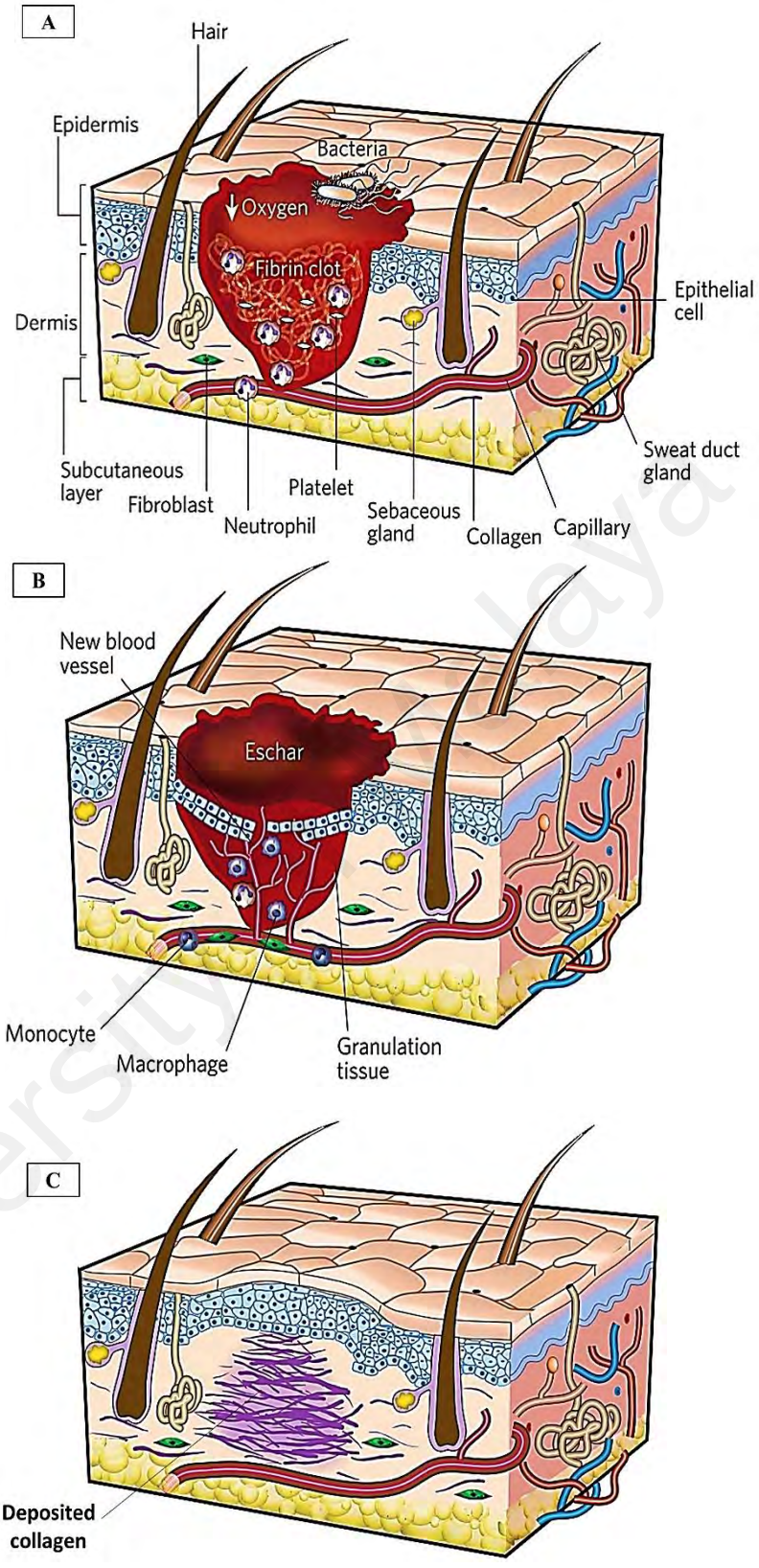


Figure 0.2: Standard stages of wound repair. (A) Inflammation, (B) New tissue formation, and (C) Remodelling (Adapted from Gurtner et al. (2008)). Reprinted with permission from Springer Nature.

Acute wounds are caused by trauma (that could be blunt or sharp) such as surgical cuts, gunshots and animal bite. As shown in Figure 2.3, healing of the acute wounds proceeds in the well-organized and overlapped process leading to predictable processes of inflammation, proliferation, and remodelling (Demidova-Rice et al., 2012; Singh & McNaught, 2017). In contrast, the chronic wound might occur if the injury does not heal normally, 12 weeks after the initial insult, and it is usually because of prolonged pathological inflammation (Guo & Dipietro, 2010; Han & Ceilley, 2017; Singh & McNaught, 2017).

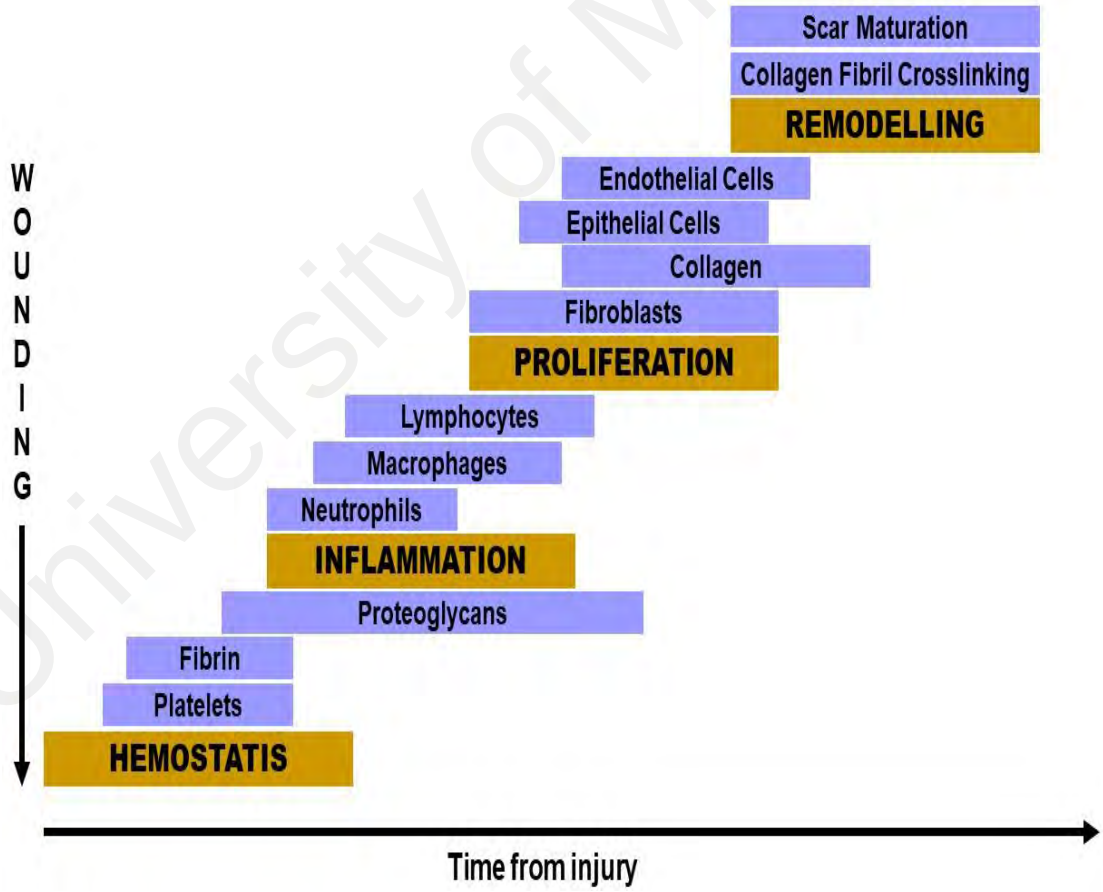


Figure 0.3: The event cascade during normal wound healing (Diegelmann & Evans, 2004). Reprinted with permission from Frontiers of Bioscience.

2.2.1 First Stage of Wound Repair: Inflammation

The immediate response after tissue damage is to avert severe loss of blood and body fluid (haemostasis), to eliminate foreign matter including dead and dying tissue, and to stop infection. Bleeding and haemostasis will happen in any wound deeper that than the epidermis since skin's blood vessel is located in the dermis and it will attract platelets (Johnstone & Farley, 2005; Singh & McNaught, 2017). The platelets undergo several physiological changes to form a platelet plug and stabilized by fibrin matrix (Gurtner et al., 2008; Periayah et al., 2017) and unleash growth factors such as platelet-derived growth factor (PDGF). The growth factor will attract white blood cells and promotes the stimulation of cytokines required for wound healing (Johnstone & Farley, 2005).

The release of chemical mediators is causing vasodilation (vascular permeability) which allows localization of neutrophils and monocytes to the wound site. Neutrophils are the 'first responders' recruited to the wound site within an hour of the insult, as shown in Figure 2.2 (A). Monocytes appear after 2–3 days of the insult, and an interplay of different cytokines (various chemical signalling mechanisms) such as activation of interleukin-1 and TGF-β signalling differentiate into macrophages. According to Wynn & Barron (2010), this process is frequently assumed as the principal regulator of wound healing inflammatory phase.

The macrophages will eliminate outside contaminant, dead or dying cells and remaining neutrophils by phagocytosis process. Besides, they also release growth factor and different cytokines to initiate tissue proliferation and cell migration, thus allowing next phase of wound healing to arise (Johnstone & Farley, 2005; Gurtner et al., 2008; Han & Ceilley, 2017). During the wound healing process, the inflammatory phase will continuously be on standby mode to ensure that all excessive bacteria, foreign material, and debris from the wound is cleared (Singh & McNaught, 2017).

2.2.2 Second Stage of Wound Repair: New Tissue Formation

The second step of wound healing is the formation of new tissue, and it occurs 2–10 days after injury. It is categorized by migration and proliferation of different cell types, as shown in Figure 2.2 (B) (Gurtner et al., 2008). Once the inflammatory response has ceased (bacteria and debris free), healing cascade involving complex processes can begin to fix the tissue damage. The complex processes include new blood vessels formation (angiogenesis), granulation tissue formation, collagen deposition, epithelization, and wound contraction, which happens simultaneously (Singh & McNaught, 2017).

Accumulation of macrophages at the injury site release chemical mediators such as PDGF and fibroblast growth factor (FGF) that attract fibroblast to the wounding area. The proliferation of fibroblast subsequently produce proteins such as collagen, elastin, and fibronectin that replace the clot at the wound site. The wound is pinkish and known as granulation tissue. The PDGF and FGF also trigger angiogenesis. Thus, a vibrant vascular network of capillaries will begin to grow throughout the wound and permit oxygen and nutrient to be delivered to the healing tissue (Johnstone & Farley, 2005; Gurtner et al., 2008; Singh & McNaught, 2017).

Fibroblast synthesized collagens that provide strength to the healing tissue and crucial for the formation of a healthy scar. An adequate amount of vitamin C is essential in the synthesis of collagen as collagen depletion would cause a delay of wound healing and impair the wound strength that leads to the risk of wound dehiscence or re-opening (Gray & Cooper, 2001; Fleck & Simman, 2010).

Contraction of the wound occurs in this phase to reduce the injury surface area by pulling together the wound edges via fibroblasts initiated by macrophages, and some differentiate into myofibroblasts. Myofibroblasts are contractile cells that, over time, bring the edges of a wound together (Gurtner et al., 2008). Epithelization is the migration

of epithelial cells across the surface of the injury. The migration of cells will stop, and the epithelial cells will begin to differentiate and form the many layers of the epidermis (Johnstone & Farley, 2005).

2.2.3 Third Stage of Wound Repair: Remodelling

The third stage of wound healing is remodelling that begins 2–3 weeks after injury and can last up to a year or more. In this stage (Figure 2.2 C), the wound undergoes maturation in which the collagen and other protein deposited in the wound become gradually well-organized (Han & Ceilley, 2017; Singh & McNaught, 2017). All the processes activated after injury come to an end and stop. Most of the endothelial cells, macrophages, and myofibroblasts undergo programmed cell death (apoptosis) or withdraw from the wound site, leaving few cells and contains major collagen and other extracellular matrix proteins (Gurtner et al., 2008).

Randomly deposited immature-type III collagen is substituted by mature-type I collagen and this procedure is achieved by matrix metalloproteinases that are secreted by macrophages, fibroblasts, and endothelial cells. However, the repaired tissue will never regain the properties of healthy skin (Gurtner et al., 2008; Han & Ceilley, 2017; Singh & McNaught, 2017). The cells and growth factors associated with the inflammation is summarized in Table 2.1 and Table 2.2, respectively.

Table 0.1: Cells involved in wound healing.

Cell type	Time of action	Functions
Platelets	Seconds	- Formation of thrombus - Instigation of the coagulation cascade - Inflammatory mediators' regulator (PDGF, TGF-β, FGF, histamine, serotonin, bradykinin, prostaglandins, and thromboxane)
Neutrophils	A peak at 24 hours	- Phagocytosis of bacteria - Debridement of wound - Unleash of proteolytic enzymes - Oxygen-free radicals generator - Improve the permeability of vascular
Keratinocytes	8 hours	- Release of inflammatory mediators - Stimulate migration of neighbouring keratinocytes - Formation of new blood vessels
Lymphocytes	72–120 hours	- Regulator of cell proliferative phase - Deposition of collagen
Fibroblasts	120 hours	- Synthesis of granulation tissue and collagen - Component builder of extracellular matrix - Release of proteases and inflammatory mediators

(Adapted from Singh & McNaught (2017))

PDGF = Platelet-derived growth factor; TGF-β = Transforming growth factor; FGF = Fibroblast growth factor

Table 0.2: Growth factors involved in wound healing.

Factor	Released from	Actions
Fibroblast Growth Factor (FGF)	Macrophages Platelets	- Activated angiogenesis - Initiates proliferation of epithelial cell and fibroblasts
Transforming Growth Factor β (TGF-β)	Platelets Neutrophils Macrophages Fibroblasts	- Chemotaxis - Trans-differentiation of fibroblast to myofibroblasts - Construction of collagen matrix - Activates angiogenesis - Contraction of wound - Stimulation of matrix metalloproteinase (MMP)
Platelet-derived Growth Factor (PDGF)	Platelets Fibroblasts Endothelial cells Macrophages	- Chemotaxis - Fibroblast proliferation - Collagen deposition
Vascular Endothelial Growth Factor (VEGF)	Platelets Neutrophils Keratinocytes	- Stimulates angiogenesis - Neovascularization

[Adapted from Singh & McNaught (2017)]

2.3 Factors that Affect Wound Healing Process

In wound management, the ideal wound healing is characterized by shortening the duration of wound closures with no side effects (Biswas et al., 2017). Aberration in wound healing will lead to delayed or excessive wound healing (Wang et al., 2018). However, many factors can influence the wound healing process, and they can be categorized into local and systemic factors.

2.3.1 Local Factors

Local factors directly affect the physical appearance (size and depth) of the wound, such as oxygenation and wound infections (Johnstone & Farley, 2005; Guo & Dipietro, 2010).

2.3.1.1 Oxygenation

In chronic wound (Figure 2.4), disruption of vascular and high consumption of oxygen by active metabolic cells caused hypoxic (oxygen depletion) microenvironment of the wounding area. When this happened, healthy cells were not able to perform cell metabolism to produce energy (ATP) as the oxygen level decrease over-time of post-injury (Guo & Dipietro, 2010). Fibroblast and keratinocyte cells proliferation and migration were disrupted; thus, prevent the angiogenesis. Angiogenesis is vital to reverse tissue ischemia (poor vascular perfusion) and to aid tissue reoxygenation, ensuring successful healing of the wound (Crowther et al., 2001).

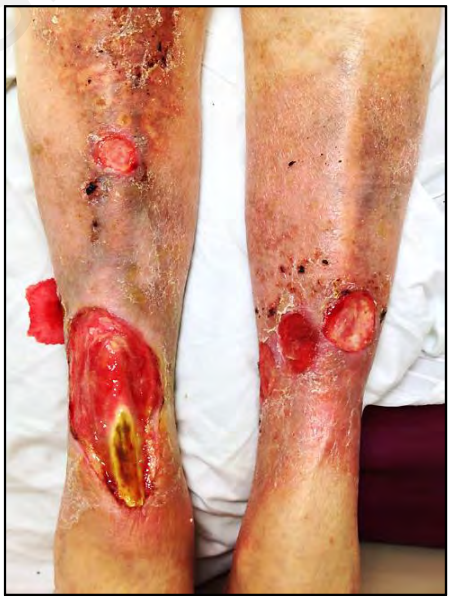


Figure 0.4: Chronic ulcers of peripheral vascular disease (Han & Ceilley, 2017). Reprinted with permission from Elsevier.

In acute wounds, hypoxia stimulates the production of cytokines and growth factors that modulate angiogenesis, promote migration and proliferation of fibroblasts such as vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), tumour necrosis factor-alpha (TNF-α), thymidine phosphorylase, and interleukin 4 (IL-4) (Crowther et al., 2001; Gurtner et al., 2008). In all, the appropriate level of oxygen is vital for optimal wound healing. Hypoxia helps to stimulate growth factors and cytokine signal molecules to promotes angiogenesis and oxygen is required for the metabolism of healthy cells to sustain the healing process.

2.3.1.2 Wound Infection

Once the skin is injured, microorganisms live on the skin surface and from the environment can infect and colonize underlying tissue. The infective process is influenced by location, depth and type of wound, level of tissue perfusion (vascular disruption), and the response of the host immune system (Bowler et al., 2001). As shown in Table 2.3, there are four groupings of infection – contamination, colonization, local infection/critical colonization, and spreading invasive infection. Elimination risk of microbial infection is vital during the wound healing process. Thus, infected wounds must be appropriately managed to reduce the microbial load because it can lead to prolonged inflammation and subsequent destruction of tissues (Maver et al., 2015).

Table 0.3: Classification of infection based on microbial replication status.

Type of infection	Microbial replication status
Contamination	Presence of non-replicating organism
Colonization	Existence of replicating microorganism on the wound (without tissue damage)
Local infection / critical colonization	An intermediate phase - replication of microorganism and the start of local tissue responses
Spreading invasive infection	Presence of replicating microorganisms inside a wound following host injury

[Adapted from Guo & Dipietro (2010)]

An infected wound will show several inflammation signs such as swelling, redness, and pain that usually associated with a foul odour (indication for dead tissue or necrosis) and affect the whole-body health condition (Johnstone & Farley, 2005). Inflammation is one of the wound healing processes to remove contamination (invading pathogenic microorganisms). If the decontamination is ineffective, inflammation may be prolonged until all microorganisms are cleared. This is because macrophages and neutrophils modulate pro-inflammatory cytokines such as interleukin-1 (IL-1) and TNF-α. Continuous inflammation will lead an acute injury to enter a chronic state consequently, fail to heal (Guo & Dipietro, 2010; Takeo et al., 2015).

Common bacteria in wounds are *Enterococcus* spp., *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus*, and *Streptococcus* spp. and they usually colonize within 48 hours after injury (Bowler et al., 2001; Agyare et al., 2013; Wong et al., 2015). *P. aeruginosa* infected the wounds by forming biofilms, which are complex communities of accumulated bacteria embedded in a self-secreted extracellular polysaccharide matrix.

The biofilms are shielding the bacteria from the phagocytic activity and further explain the failure of antibiotics in wound healing treatment (Guo & Dipietro, 2010).

There were also few cases reported on wound infection due to fungus. From a clinical report, nine post-cardiac-surgery patients were affected with *Aspergillus flavus* at A. Z. Sint-Jan General Hospital of Brugge (Belgium) from 18 January 1998 through 17 April 1998 (Vandecasteele et al., 2002). Besides wound infection, cutaneous aspergillosis is a rare form of locally invasive fungal infection among both immunocompetent and immunocompromised host. *Aspergillus fumigatus*, *A. flavus*, and *A. terreus* are the common cause of aspergillosis and rare case caused by *Aspergillus niger*. An incident on primary cutaneous aspergillosis-Tinea pedis (fungal infection of the soles of the feet) caused by *A. niger* was reported in the city of Kancheepuram district, India. The hyper-pigmented plaque-like scaly lesion was seen over the patient's right foot (Figure 2.5) and from a clinical and cultural examination, the fungus was identified as *A. niger* (Prasanna et al., 2016).

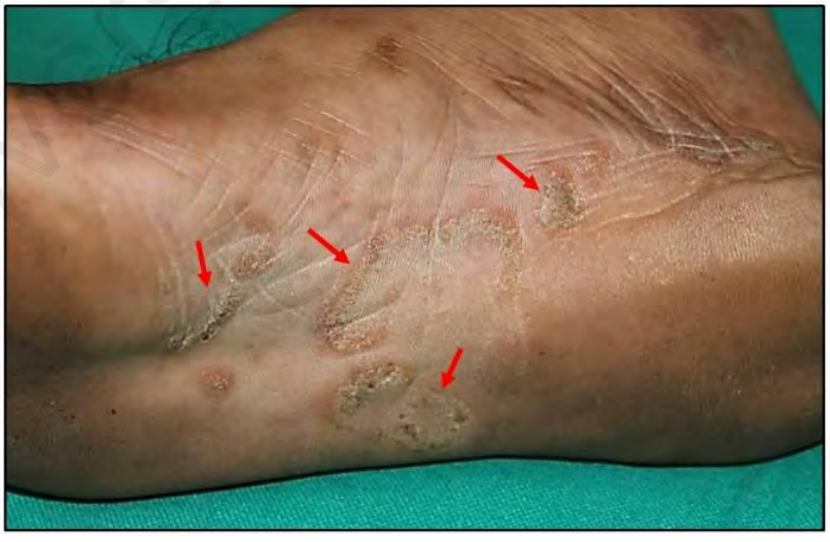


Figure 0.5: Primary cutaneous aspergillosis-Tinea pedis caused by *A. niger*. Hyper-pigmented plaque-like scaly lesion (shown by the red arrow) can be seen on the patient's foot (Prasanna et al., 2016). Reprinted with permission from International Journal of Advanced Research (IJAR).

2.3.2 Systemic Factors

Systemic factors are the general health factors of the individual that influence the ability in wound healing (Johnstone & Farley, 2005; Guo & Dipietro, 2010). These include age of patient, immunocompromised condition, and lifestyle. Several studies reported that older skin is slower to heal because of slow inflammatory response such as altered chemokine production, delayed T-cell infiltration into the wounded site, and reduction of phagocytic action of macrophages (Swift et al., 2001; Gosain & DiPietro, 2004). Another factor that affected wound healing is immunocompromised conditions, especially in HIV/AIDS patients. According to the World Health Organization (2019), Human Immunodeficiency Virus (HIV) weakens human immune and defence system (by depletion of CD4 T-cells). While Acquired Immunodeficiency Syndrome (AIDS) is the advanced stage of HIV infection that takes about 2–15 years to develop (depend on individual). The immune system in patients with HIV is progressively deteriorated, which can lead to harmful pathogenic infections and delayed wound healing (Singh & McNaught, 2017).

Unhealthy lifestyle such as alcohol consumption and smoking affects wound healing process (Guo & Dipietro, 2010). Previous studies reported that chronic alcohol intake and unhealthy diet were associated with impeding wound healing by showing incomplete re-epithelialization, delayed in wound contraction and wound closure (Ranzer et al., 2011; Rosa et al., 2017). Patients with diabetic foot ulcers who smoke, their wounds are hard to be properly healed because smoking caused tissue hypoxia, impaired migration of white blood cells and macrophages into the wounding site, and reduced proliferation and migration of fibroblasts (Ahn et al., 2008; Xia et al., 2019).

2.4 Wound Therapeutic Agent from Natural Resources

Medicinal plants have been used as a therapeutic agent and being documented since ages (Kinghorn et al., 2011). Over the years, many drugs discoveries for a diverse range of disease are derived from natural sources such as plants, marine organisms, terrestrial animals, and microbes (Newman & Cragg, 2016). Today, natural products remain to play a significant role in the drugs development process

The oldest written evidence, approximately to be 5000 years old, was found on a Sumerian clay slab from Nagpur, India. It contains medicinal plants' usage including 12 recipes for the preparation of drugs referring to over 250 various plants. Other historical medicinal plants reference such as the *Pen T'Sao* (Chinese book on roots and grasses written by Emperor Shen Nung circa 2500 BC), the *Vedas* (The Indian holy books which mention about the Ayurveda medical system) and the *Ebers Papyrus* (written circa 1550 BC, contains a collection of 700 plant species for remedy such as pomegranate, aloe, onion, fig, willow etc.) (Petrovska, 2012).

2.4.1 Plants for Wound Healing Treatment

According to Dan et al. (2018), there are three main factors for choosing plants as a potential wound healing agent. First, almost 25% of therapeutic drugs are plant-based or derived from plants. Second, the researchers are discovering alternative and cost-effective therapeutics since wound treatment is bearing huge financial burden towards the patients. Finally, current reports on drug resistance against pathogenic agents had set the alarm and demanding for novel antimicrobial therapies. The selection of medicinal plants as an alternative method of wound treatments was made base on the availability and affordability of the source. Table 2.4 provides a summary of the widely used therapeutic plants for the treatment of wound healing.

Table 0.4: Overview of the widely used medicinal plants for wound healing treatment.

Plant	Common name	Plant part used	Description	References
Aloe vera	Aloe vera	Mucilage from the inner leaf	Several reported bioactivities, such as antibacterial, antioxidant, anti-inflammatory, and wound healing. Enhance production of collagen, fibroblast growth factors, fibroblast proliferation, and wound contraction. Identified active compound for wound healing properties is glucomannan (a type polysaccharides).	(Schmidt & Greenspoon, 1991; Hashemi et al., 2015)
Azadirachta indica	Neem	Seed oil, bark, and leaves	Traditionally used for various ailments such as asthma, intestinal worms, skin ulcers and cure for fever. Reported biological activities: anti-inflammatory, antiulcer, antifungal, antibacterial, antioxidant, anticancer, and immunomodulatory. Neem oil increase production of collagen that maintains skin elasticity and accelerates cell proliferation phase during the wound healing process. Identified active compounds for wound healing properties are nimbidin, nimbin, and nimbidol.	(Paul et al., 2011; Ilango et al., 2013; Chundran et al., 2015)

Table 2.4, continued

Curcuma longa	Turmeric	Root	*C. longa* is one of the spices used in cooking, and it is topically applied to wounded skin. Turmeric is reported to have antifungal, anti-inflammatory, antibacterial, and analgesic activities. Vitamin A and proteins in the turmeric were able to induce the production of collagen. Curcumin is the active compound found in the turmeric that enhances the formation of granulation tissue, deposition of collagen, remodelling of tissue, and fasten wound contraction.	(Kundu et al., 2005; Maliheh et al., 2014; Tejada et al., 2016)
Calendula officinalis	Pot marigold, marigold	Flower	Calendula flowers have been used in the form such as infusions, liquid extracts, ointments, and cream for the treatment of dermatological disorders. Ethanolic and hexane extract of the flowers were able to enhance the proliferation and migration of fibroblasts by modulating inflammatory phase through activation of transcription factor NF-κβ and production of more IL-8 chemokine. The presence of phytochemical compounds such as flavonoids (rutin and quercetin), amino acids, and polyphenols may be responsible for the wound healing activity.	(Leach, 2008; Fronza et al., 2009; Dinda et al., 2015; Nicolaus et al., 2017)

2.4.2 Vegetables and Wound Healing

Traditional vegetables used by the different ethnics in Malaysia include more than 120 species representing numerous families, from shrubs to huge trees. The leaves, shoots, or rhizomes of the vegetables are eaten fresh as a salad or cooked (Abas et al., 2006). In many Asian countries, consumption of raw vegetables is deemed as a traditional healthy diet since the diet is rich in antioxidant agents such as chemical compounds, vitamins, enzymes, minerals, and fibres valuable to health (Ong, 2004; Sulaiman et al., 2011).

A popular local vegetable, *Portulaca oleracea* (common name: purslane), has been used as a traditional remedy in various countries to treat countless illnesses in humans (Chan et al., 2000) and a recent study indicated that the extract of *P. oleracea* accelerates wound healing on indomethacine and phenylbutazone-induced ulcers (Rashed et al., 2003). *Carica papaya* L. (common name: pawpaw, papaya) is commonly cultivated in tropical countries. The leaves are edible and used traditionally as a dressing to treat wounds (Burkill, 2000). In Ghana, *Momordica charantia* L. (common name: bitter gourd) was used in folklore to treat injuries, ulcer, fever, piles, and skin infections (Agyare et al., 2009).

2.5 Description and Therapeutic Values of the Selected Vegetable Species

Current devotion is being drawn towards discovering plant sources for substances that offer nutritional and pharmaceutical benefits to human (Raju et al., 2007). Literature survey revealed that numbers of traditional vegetables in Malaysia are used for both as food and medicine, which is used raw, boiled, or applied as a poultice (Andarwulan et al., 2010). *Acalypha indica* L., *Centella asiatica* (L.) Urb., and *Sesbania grandiflora* (L.) Pers. are locally available vegetables that are widely consumed by the Asian people.

These plants have been used in traditional practices for wound healing properties and recorded for other therapeutic values.

2.5.1 *Acalypha indica* L.

Acalypha indica L. (Family: Euphorbiaceae) is commonly known as Indian copperleaf, India acalypha and locally known as *Kucing Galak* and *Cika Mas*. It is a renowned traditional plant, particularly in Asia and Africa. The plant can also be found in most wet, temperate, tropical countries in Asia, Europe, and both South and North American regions. It grows as a weed in backyards, bushes, alongside roads, and other places (2016b; Zahidin et al., 2017).

A. indica is an annual, erect herb that can grow up to 1 m high, and short life span (about 40 days) (Venkatachalam et al., 2017). The leaves are 2.5–7.5 cm long with 2.0–4.5 cm broad either ovulate or rhombic ovulate shape, simple and arranged spirally. The flower is arranged in numerous lax, erect, elongated, auxiliary spikes, and clusters near the summit of the spikes, as shown in Figure 2.6.

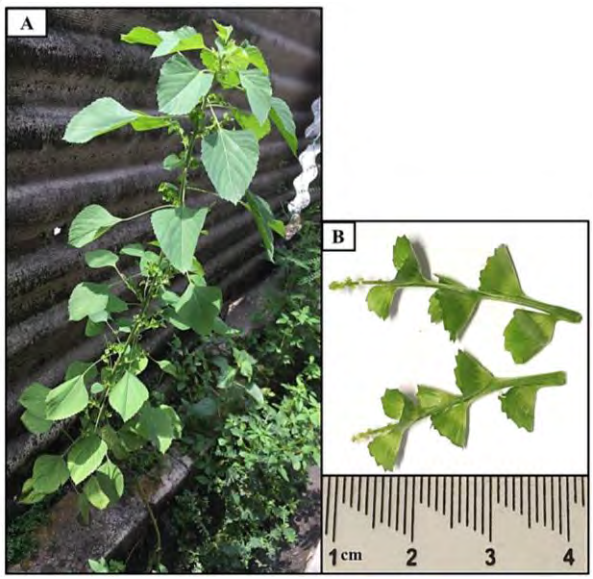


Figure 0.6: *A. indica* usually grows as weed. (A) Whole plant, (B) Flowers.

A. indica is widely used in the preparation of medicine based on their traditional belief in various regions and countries (Table 2.5). The whole parts of this vegetable, including roots and shoots, can be used as a herbal remedy to heal various diseases.

Table 0.5: Ethnomedicinal used of *A. indica*.

Country	Plant parts	Medicinal uses	References
India	Leaves	Mixed with oil for various dermatology ailment	Lingaraju et al. (2013)
	Roots	Laxative and blood dysentery, fever, and wound healing	Basha & Sudarshanam (2011); Mohan et al. (2012)
Malaysia	Whole parts	Treat mouth ulcer	(2016b); (Zahidin et al., 2017)
	Leaves	Added with turmeric for alleviation from skin break out and pimples	
Mauritius	Whole parts	Skin infection such as scabies and dermatitis	Rahman et al. (2010)
	Roots	Treat ear infection	(Seebaluck et al., 2015)
Nepal	Leaves	Burn and rheumatoid arthritis	(Singh et al., 2012)

In recent years, various scientific studies were done to investigate multiple pharmacological properties of the *A. indica*. Rahman et al. (2010) showed that orally administered methanol extract of *A. indica* significantly reduces writhing reflexes in Swiss albino mice by 51.1% (dose: 200 mg/kg) and 57.2% (400 mg/kg) within 10 minutes of administration of acetic acid. Methanol extract from the aerial part of *A. indica* exhibited cytotoxicity against human small cell lung NCI-H187 cancer with an IC_{50} value of 25 µg/ml (Sanseera et al., 2012). Methanolic extract of *A. indica* stems was able to control postprandial hyperglycaemia (rise in the level of blood glucose) by inhibiting the α-glucosidase enzyme in maltose and sucrose leaded diabetic rats (Priya & Rao, 2016).

This vegetable has been commonly used in traditional practices to treat various skin problems including as wound healing. Besides, many researches were performed to investigate the traditional claims, to provide scientific evidence of the claims, and to explore potential therapeutic advantages of the plant. Thus, *A. indica* was chosen for this study.

2.5.2 *Centella asiatica* (L.) Urb.

Centella asiatica (L.) Urban, a clonal, perennial herbaceous creeper (Figure 2.7) belonging to the family Umbelliferae (also known as Apiaceae) is found throughout India, Sri Lanka, Malaysia, and other parts of Asia (Govarthanan et al., 2015). Commonly known as Asiatic pennywort, Indian pennywort, *Gotu Kola*, and *pegaga*, the vegetable has been used since prehistoric times, particularly in the Ayurvedic medical system of India and the folk remedy of China. It is recommended by the World Health Organization (WHO) as one of the most important therapeutic plant species to be conserved and cultivated. In Malaysia, even though the traditional healer has used it in their herbal remedies, it is more popular as a traditional vegetable or an *ulam* specifically among the Malay communities rather than a therapeutic plant (2016a).

Figure 0.7: *C. asiatica* is a herbaceous creeper usually found growing as floor herbs.

Therapeutic values of *C. asiatica* are well known among Asian old-folklore practices. It has been using to treat kidney problem, bronchitis, skin diseases, asthma, gastric, and mental illness (Ruszymah et al., 2012; Govarthanan et al., 2015). In Malaysia, *C. asiatica* is widely consumed raw as *ulam* by the Malay community. The Chinese and the Indians consumed it as cooling drink and brain tonic, respectively. In Malay-traditional medicine,

the decoction of leaves is used to cure leprosy, rheumatism, and diarrhoea in children. The leaves are ground into a paste to reduce fever. In postnatal care for women after giving birth, the leaves are used to preserve youthfulness (2016a). Besides all the traditional claims, many scientific studies (Table 2.6) was done to investigate various potential pharmaceutical activities of *C. asiatica*.

Table 0.6: Scientific studies of potential pharmaceutical activities of *C. asiatica*.

Bioactivity	A brief description of the study	References
Anticancer	Acetone extract displayed significant cytotoxicity against human breast MCF-7 cancer cells (IC_{50} = 53.7 ± 0.06 µg/ml) and human lung A549 cancer cells (IC_{50} = 46.0 ± 0.04 µg/ml).	(Soyingbe et al., 2018)
	Asiatic acids inhibit proliferation, migration, and induce apoptosis of SW480 and HCT116 colon cancer cells by modulating phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt)/mammalian target of rapamycin (mTOR) and p70S6K signalling pathway.	(Hao et al., 2018)
Antiulcer	Albino rats of Charles Foster strain were orally administered with ethanol extract (roots) showed a smaller number of ulcers (15.0 ± 2.6), comparable with Diazepam (positive control) that showed 27 ulcers.	(Sarma et al., 1995)
Cardio-protectivity	Aqueous extract of *C. asiatica* demonstrated a cardio-protective effect in adriamycin-induced cardiac damage in Winstar rats.	(Gnanapragasam et al., 2004)
Neuroprotective effects	*C. asiatica* protects in rats induced with D-galactose and aluminium chloride ($AlCl_3$) by decreasing the level of acetylcholinesterase (AChE) and improvement of cognitive impairment.	(Lokanathan et al., 2016; You et al., 2018; Chiroma et al., 2019)
Hepatoprotective effects	Rats were orally administered with aqueous extract of *C. asiatica* (100 or 200 mg/kg) showed significant decrease level of malondialdehyde, increase the level of antioxidant enzymes, and reduced level of pro-inflammatory mediators. Assessment of liver histology showed that the extract alleviates mass of tissue necrosis.	(Choi et al., 2016)

2.5.3 *Sesbania grandiflora* (L.) Pers.

Sesbania grandiflora from family Fabaceae (also known as Leguminosae) was commonly known as *agathi* or hummingbird is one of the significant plants used in old-folklore remedy and consume as vegetables in India (Sinha & Lakra, 2005; Das et al., 2013). This plant is local to tropical Asia, commonly found in Malaysia (locally known as *turi* and *geti*), India, Indonesia, and the Philippines. It is a small branching tree that can grow up to 8–15 m tall and 25–30 cm in diameter (Figure 2.8). The flowers are white, red, or pink and available throughout the year, mainly during the winter season (China et al., 2012; Hasan et al., 2012).

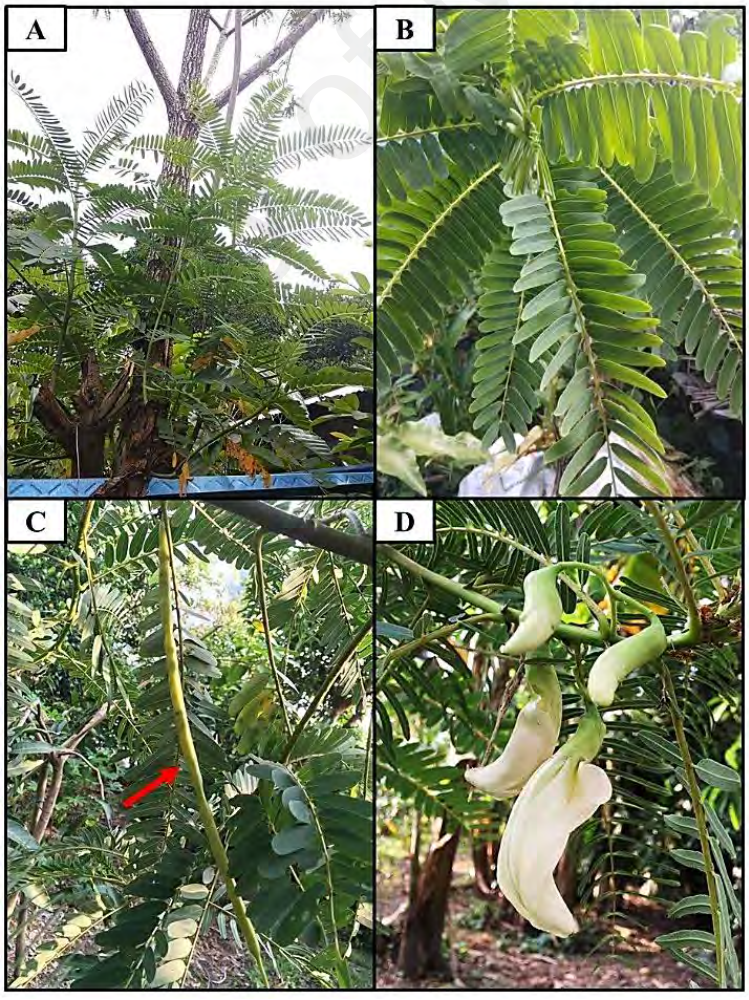


Figure 0.8: (A) *S. grandiflora* tree, (B) Leaves of *S. grandiflora*, (C) Long, thin green beans of *S. grandiflora* (shown by the red arrow), (D) Flowers of *S. grandiflora*.

In Ayurveda and South Eastern Asia medicine, whole parts of the plant were fully utilized to treat different diseases. The root poultice is applied to reduce inflammation, sore spots, and fever (Sreelatha et al., 2011). The leaves are used for the treatment of epilepsy, ulcer, night blindness, and applied to sprains or bruises on the body. Tea prepared from dried leaves is claimed to have anthelmintic, antibiotic, anti-tumour, and contraceptive properties. However, over consumed of the leaves might cause diarrhoea. The flower is used as an antiseptic as well as for pain-relieving. The bark is said to have antipyretic properties and a remedy for gastric pain (Sheikh et al., 2011; Sreelatha et al., 2011; Hasan et al., 2012). Despite the ethnomedicinal claims, not many scientific studies on the bioactivity of *S. grandiflora* are reported. Table 2.7 shows the scientific findings of *S. grandiflora*.

Table 0.7: Scientific findings related to the bioactivity of *S. grandiflora*.

Bioactivity	Description	References
Anticancer	Ethanol extract of flowers and leaves (doses of 100 and 200 mg/kg) exhibited anticancer activity against Ehrlich ascites carcinoma (EAC)-bearing Swiss albino mice.	(Sreelatha et al., 2011)
	The methanol extract (flowers) showed cytotoxicity against human cervical cancer HeLa cell line (IC_{50} value of 0.13 mg/ml).	(Loganayaki et al., 2012)
Anti hyperglycemic	Aqueous extract from seeds of *S. grandiflora* (2.5 g/kg) decreases the level of blood glucose (435 mg/dl to 213 mg/dl) within 18 days of treatment.	(Zamroni et al., 2017)
Anti tuberculosis	Isovestitol, medicarpin, and sativan isolated from the methanol extract of *S. grandiflora* (root) showed antituberculosis activity against *Mycobacterium tuberculosis* H37Rv, with MIC values of 50 µg/ml.	(Hasan et al., 2012)
Anti-ulcer	At concentration 100 and 200 mg/kg, hydroalcoholic extracts (leaves) exhibited a protective effect in acetic acid-induced ulcerative colitis in mice via antioxidant mechanism and anti-inflammatory activities.	(Gupta et al., 2018)
Hepato-protective	Ethanol extract (250 and 500 mg/kg) and aqueous extract (500 mg/kg) of flower significantly improved the function of hepatocytes in carbon tetrachloride (CCl_4) induced liver damage in Swiss albino rats.	(Kale et al., 2012)
Phyto-remediation	Roots of *S. grandiflora* were able to accumulate 118 mg/g of lead (Pb). Heavy metal tolerance mechanism via increase level of antioxidant enzymes, photosynthetic enzymes, and malondialdehyde (MDA) content.	(Malar et al., 2014)

2.6 Polyherbal

Development of polyherbal therapeutic drugs and synergy research is an integral part of the phytomedicine field (Yuan et al., 2017). Polyherbal holds few advantages such as effective in a vast number of diseases, wide therapeutic range (effective at a low dosage and safe at high dosage), fewer side effects, eco-friendly, and readily available compared to allopathic drugs. There are two principles in Ayurveda and Traditional Chinese Medicine (TCM) of herbal formulation to cure and treat diseases – use of a single drug and use of more than one drugs (polyherbal) (Li et al., 2010; Parasuraman et al., 2014; Jaiswal et al., 2016).

Formulation of TCM was based on six modes of polyherbal interactions such as counteraction, potentiation, reinforcement, restraint, detoxification, and toxicity (summarized in Table 2.8) (Che et al., 2013; Zhou et al., 2016). It is important to understand the modes of action for each combination for the development of safe and effective herbs combination. Table 2.9 showed some of the work on synergistic effects of the herb-herb combination.

Table 0.8: Basic modes of herb-herb interactions in Traditional Chinese Medicine

Modes	Interactions	Example	Description
Counteraction	Antagonistic - Therapeutic effect of a herb is weakened by another herb	Interaction between turnip root/seed and ginseng root	Ginseng is a tonic to increase vital energy. But the effect was reduced or even eliminated with the presence of turnip.
Potentiation	Two herbs used in the same prescription. One herb serves as the principal herb and the other as an adjunct or auxiliary herb	Combination of ginseng root and aconite root (*Aconitum carmichaelii*)	Ginseng is the main component of strength and vitality. While aconite aids to warm the body and raise the *yang* energy. Both herbs can replenish and restore vital physiological functions, particularly during emergency condition such as collapse and shock.
Reinforcement	Herbs with similar medicinal properties used together to produce greater efficacy	Combination of rhizomes of *Carydalis yanhusuo* and *Curcuma phaeocaulis*	Both herbs are valuable for enhancing circulation of blood, alleviating blood stasis, and relieving pain.
Restraint and detoxification	Usage of toxic herbs with other herbs to make the poisonous effects more tolerable (antidote and reduce/neutralize the toxicity)	Combination of ginger and pinellia (*Pinellia ternata*) tuber	*Pinellia* tuber is a toxic herb (cause mucosal irritation and inflammation in the throat and gastrointestinal tract). It is usually cooked with ginger to decrease the toxicity of *Pinellia.*
Toxicity	Interaction of herbs that lead to unfavourable outcome such as toxic and other side effects	- *Glycyrrhiza / Daphne genkwa* - *Glycryyhiza / Euphorbia kansui* - *Sophora flavescens / Veratrum nigrum*	The herbs are mutually incompatible and must be avoided in any prescription.

[Adopted from Che et al. (2013)]

Table 0.9: Combination of herbs for the treatment of various diseases.

Combination of extracts	Bioactivities	References
Ethanol-based mixed extract of *Curcuma longa*, *Areca catechu*, *Oryza sativa*, and *Garcinia mangostana*	Low toxicity and excellent wound healing properties	(Chusri et al., 2013)
A mixture of honey and *Nigella sativa* oil (ratio 1:1)	Topical application of the mixture accelerate wound healing on rats	(Javadi et al., 2018)
A mixture of *Hypericum perforatum*, *Agrimonia eupatoria*, and *Satureja hortensis*	Antibacteria properties against *S. aureus* and *P. aeruginosa*	(Avci & Gergeroglu, 2019)
Herbal blend of *C. asiatica* and *Vitis vinifera*	Antioxidant and protectivity against H_2O_2	(Jeon et al., 2020)
Aqueous extract of *Bergenia ciliate*, *B. stracheyi*, *Rumex dantatus*, and *R. hastatus*	Antibacterial activities against *S. aureus* and *P. aeruginoasa*	(Rashid et al., 2019)
Combination of *A. indica* and *C. asiatica*	Decrease neuronal damage in hypoxia-induced hippocampal injury in rats	(Farida et al., 2018)
Combination ofof selected herbs: coriander and cumin (1:1) coriander and mustard (1:1) cumin and mustard (1:1)	DPPH free radical scavenging and antibacterial properties	(Bag & Chattopadhyay, 2015)
Combination of *Salacia chinensis, Curcuma longa*, and *Tinospora cordifolia*	DPPH and H_2O_2 free radical scavenging and FRAP reducing activities	(Choudhari et al., 2020)

METHODOLOGY

3.1 Preparation of Vegetable Extracts

3.1.1 Solvents

Methanol was purchased from Fisher Scientific, UK. Analytical grade organic solvent was used for the extraction processes of all the vegetables.

3.1.2 Plant Collection

Acalypha indica L. was collected from Taman OUG, Kuala Lumpur in March 2017; *Centella asiatica* (L.) Urb. was collected from Rimba Ilmu Botanic Garden, Universiti Malaya in December 2018; and *Sesbania grandiflora* (L.) Pers. was collected from Muar, Johor in November 2018. These specimens were authenticated at the KLU herbarium by Dr. Sugumaran Manickam.

3.1.3 Preparation of Vegetable Extracts

3.1.3.1 Aqueous Extraction

Fresh leaves (20 g) of *A. indica* (AI-A), *C. asiatica* (CA-A), and *S. grandiflora* (SG-A) were cut into pieces of about 1 mm^2 and heated separately at 80ºC in 500 ml of distilled water for 1 hour. The solutions were filtered through filter paper (Whatman No.1), and the aqueous filtrates were collected, freeze-dried, and stored in the dark at 4ºC until further use (Krishnan, 2005; Giribabu et al., 2020).

3.1.3.2 Methanol Extraction

The leaves of *A. indica* (AI-M), *C. asiatica* (CA-M), and *S. grandiflora* (SG-M) were oven-dried at 35ºC for 3–5 days, ground into powder (40 g), and soaked in methanol (MeOH) at 1:10 (w/v) for 72 hours at room temperature. The filtrates were collected, and excess methanol was evaporated at 40ºC under reduced pressure using a rotary evaporator. The extracts were stored at −20ºC until further use (Azis et al., 2017).

3.1.3.3 Preparation of Single and Combination Solutions of Extracts

The stock solution (20 mg/ml) of the aqueous extracts was dissolved in sterilized distilled water, filtered through a 0.2 µm filter cap (Orange Scientific, Belgium). Methanol extracts were dissolved in sterile distilled water and filtered via filter paper (Whatman No.1). Combination solutions of the extracts were prepared at 1:1 ratio (v/v) from the aqueous and methanol stock solutions. The 1:1 ratio was selected because it was the standard ratio or proportion used widely in the preparation of herbs/medication in traditional medicine and the ideal starting point in the investigation of combination efficacy before proceeding the experiment in different ratio of combination (Chou, 2010; Sulekha et al., 2017). The extracts were prepared at various concentrations needed by each experiment by serial dilution of the stock solutions (Krishnan, 2005; Azis et al., 2017). Table 3.1 shows the abbreviation of the single and combined vegetable extracts used in this study.

Table 0.1: Abbreviation of the single and combined vegetable extracts.

	Abbreviation	Vegetable extracts
Single vegetable extracts	AI-A	Aqueous extract of *A. indica*
	CA-A	Aqueous extract of *C. asiatica*
	SG-A	Aqueous extract of *S. grandiflora*
	AI-M	Methanol extract of *A. indica*
	CA-M	Methanol extract of *C. asiatica*
	SG-M	Methanol extract of *S. grandiflora*
Combined vegetable extracts (1:1)	AI-A+CA-A	Aqueous extract of *A. indica* and *C. asiatica*
	AI-A+SG-A	Aqueous extract of *A. indica* and *S. grandiflora*
	CA-A+SG-A	Aqueous extract of *C. asiatica* and *S. grandiflora*
	AI-M+CA-M	Methanol extract of *A. indica* and *C. asiatica*
	AI-M+SG-M	Methanol extract of *A. indica* and *S. grandiflora*
	CA-M+SG-M	Methanol extract of *C. asiatica* and *S. grandiflora*

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), penicillin (100 U/ml), streptomycin (100 µg/ml) and amphotericin B (250 µg/ml) were purchased from PAA Lab, Austria. Accutase in DPBS, 0.5 EDTA was purchased from iCT, California, USA. HEPES was obtained from Molekula, UK. Sodium bicarbonate ($NaHCO_3$) was obtained from R & M Chemical, UK. Neutral red was purchased from ICN, Ohio, USA. All other chemicals and solvents used were of the highest purity, and grade available purchased from BDH AnalaR, UK and Sigma-Aldrich, USA. Cell culture plasticware was from Orange Scientific, Belgium.

3.2.2 Cell Culture

The normal human dermal fibroblast NHDF cell line (PCS-201-012) was purchased from American Type Culture Collection (ATCC) (Virginia, USA). NHDF cells were cultured in DMEM with 10% (v/v) Foetal bovine serum (FBS), 100 U/ml of penicillin, 100 µg/ml of streptomycin, and 200 µg/ml of amphotericin B. Cells were maintained in T25 flasks (Nunc, Denmark) with 10 ml of media and incubated at 37ºC and 5% CO_2. NHDF cells that were grown to 80–90% confluence and passages 6–7 were used in the experiments (Kim et al., 2016).

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

The effects of aqueous and methanol vegetable extracts on NHDF cells were evaluated by NRU assay (Ramasamy et al., 2012; Mahdzir et al., 2017). Detachment of the NHDF cells from T25 flask was done by adding 1 ml of accutase and 3 ml of phosphate buffer solution (PBS) pH 7.4. The cell suspension was centrifuged at 100 ×g for 5 minutes (Kubota 2010, Japan) to obtain a cell pellet. The supernatant was discarded, and the cells were resuspended in DMEM with 10% FBS. Trypan blue (0.4%, w/v) exclusion method was used to count the number of viable cells (Freshney, 1994; Crowley et al., 2016).

NHDF cells at 3×10^4 cells/ml were seeded into 96-well plate in DMEM supplemented with 10% FBS for 24 hours to allow the cells to adhere. Then, all the single and combined vegetable extracts (25, 50, 75, and 100 µg/ml) were added into each well and was incubated for another 48 hours. Untreated NHDF cells (without the addition of vegetable extracts) was used as a negative control. After the 48 hours incubation period, the medium was discarded and replaced with 50 µg/ml NR solution. The 50 µg/ml NR solution was prepared in the culture media and incubated in the dark for 24 hours prior use. The plate was incubated for another 3 hours to permit uptake of the vital dye into the lysosomes of

uninjured and viable cells. The medium was discarded, and a mixture of calcium chloride-formaldehyde was used to rinse the cells. Then, a mixture of ethanol, water, and acetic acid (50:49:1) was added into each well to elute the dye within the viable cells. Measurement of optical density at 540 nm was performed by using a microplate reader (Thermo Scientific, USA) and percentage of inhibition was calculated [($OD_{control}$ − OD_{sample}) / $OD_{control}$) × 100%]. The experiment was done in triplicate with standard deviation.

3.2.4 Wound Scratch Assay

The wound scratch assay procedure was carried out as described previously with slight modifications (Liang et al., 2007; Kim et al., 2016). *In vitro* wound healing analysis in NHDF cells were carried out using the single and combined vegetable extracts at a concentration of 25 µg/ml. The concentration (25 µg/ml) was chosen because the NHDF cells showed the highest percentage of cell viability based on the assessment by using the NRU assay. NHDF cells were seeded (1×10^5 cells/ml) in a 24-well plate and cultured in DMEM with 10% FBS before incubating for 24 hours to allow the formation of a confluent monolayer cell (37ºC, 5% CO_2). Next, the medium was discarded and fresh DMEM with 1% FBS was added to each well.

The cells were further incubated for another 24 hours. Then, a small linear scratch was created in the confluent monolayer cells using a sterile pipette tip. The media was removed, and cells were rinsed with phosphate buffered solution (pH 7.4) to remove cellular debris. Fresh DMEM 1% FBS and 25 µg/ml of vegetable extracts (AI-A, CA-A, SG-A, AI-M, CA-M, SG-M, AI-A+CA-A, AI-A+SG-A, CA-A+SG-A, AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M) were added to each well. DMEM 10% was used as a positive control and DMEM 1% (without addition of any extracts) was used as a negative

control. DMEM 10% provided optimal nutrients and growth factors, while DMEM 1% provided minimum nutrients and growth factors for cell cultures.

The distance of fibroblast cells migration towards the gap in the artificial wound was observed microscopically at 0, 24, and 48 hours. The percentage of wound closure was calculated according to the following formula:

$$\text{Percentage of wound closure (\%)} = \frac{\text{Distance at T0} - \text{Distance at T24 or T48}}{\text{Distance at T0}} \quad (3,1)$$

Images of migrated cells were captured using a digital camera attached to an inverted microscope (Leica DMI 3000 B) and a Leica microsystem (Leica Application Suite, LAS V4.0). The experiment was performed in triplicates.

3.3 Antimicrobial Activity

3.3.1 Antibacterial Activity

3.3.1.1 Microbial Strains and Condition

The bacterial tested for antimicrobial activity in this study were *Bacillus cereus*, *Bacillus subtilis*, *Escherichia coli*, *Micrococcus luteus*, *Serratia marcescens*, *Staphylococcus aureus*, and *Staphylococcus epidermidis.* All bacteria were obtained from the Microbiology Department, Institute of Biological Sciences, Universiti Malaya, Kuala Lumpur, Malaysia. Bacterial strains/stock were kept at 4ºC.

3.3.1.2 Kirby Disc Diffusion Assay

Antibacterial activity was determined using the Kirby–Bauer disc diffusion assay (Wan-Mohtar et al., 2016). Nutrient agar (NA) (Thermo Scientific-Oxoid, UK) medium was prepared, and 20 ml of the medium were poured into each petri dish. At least three well isolated colonies of the test organism were touched with sterile wire-loop, and the growth was transferred to a tube containing 5 ml of nutrient broth. The broth cultures were incubated at 37ºC for 24 hours, and the turbidity was adjusted to 0.5 McFarland standards (approximately 1–2 × 10^8 cfu/ml). Once the NA medium hardened, 200 µl of test bacteria suspension was smeared on the prepared agar.

Disc diffusion testing was carried out using sterile 6-mm discs (Whatman, UK). Discs were prepared using 50 µl of each vegetable extract diluted in sterile distilled water to a concentration of 200, 400, 600, or 800 mg/ml. The prepared discs were placed on the surface of the inoculated NA media and incubated at 37ºC for 24 hours. Vancomycin (30 µg/disc, Thermo Scientific-Oxoid, UK) was used as positive control while the sterile empty disc was used as a negative control. After overnight incubation, the plate was observed for zones of inhibition, which were measured using digital callipers (Taemchuay et al., 2009). Tests were performed in triplicates, and the mean of the diameters of the inhibition zones was calculated. Inhibition zones that were higher than 6 mm were considered positive for antimicrobial reactions.

3.3.2 Antifungal-demelanizing Activity on *Aspergillus niger*

3.3.2.1 Antifungal Activity

Aspergillus niger was obtained from the Microbiology Department, Institute of Biological Sciences, Universiti Malaya, Kuala Lumpur, Malaysia and used for screening assay study. *A. niger* was maintained on Potato Dextrose Agar (PDA) (Sigma-Aldrich, Dorset UK) and stored at 4°C. The antifungal-demelanizing activity of single and combined vegetable extracts was evaluated using the micro-dilution technique (Heleno et al., 2013; Wan-Mohtar et al., 2017) with slight modification. Single and combined vegetable extracts were dissolved in sterile distilled water (3.13, 6.25, 12.5, 25.0, 50.0, and 100.0 mg/ml) and added to potato-dextrose broth (Oxoid, UK) containing fungal spore suspensions. Samples were visualised by light microscope (Leica DM1000) and the lowest concentration of vegetable extracts that resulted in no visible growth (less formation of filament or mycelium) compared to the original inoculum, was defined as the MIC (Heleno et al., 2013; Wan-Mohtar et al., 2017).

Fungicidal concentrations (MFCs) were identified using a serial sub-cultivation of 2 µl of vegetable extracts dissolved in the medium for 72 hours of cultivation and subsequently transferred into 96-well plates containing 100 µl of broth per well. The plates were incubated for 72 hours at 28°C. Samples were visualised by light microscope (Leica DM1000), and the lowest concentration of vegetable extracts that resulted in no visible growth (no formation of filament or killing 99.5% of the original inoculum) was defined as the MFC. Fluconazole (2 mg/ml) was used as a positive control, and DMSO 5% was used as a negative control in these assessments (Wan-Mohtar et al., 2017).

3.3.2.2 Demelanizing Activity

The demelanizing activity of single and combined vegetable extracts against *A. niger* was evaluated in 6-well plates to determine the minimum demelanizing concentration (MDC). Treated and untreated *A. niger* cultivations were observed by light microscope (Leica DM1000). DMSO 5% was used as a negative control. The lowest concentration of vegetable extracts that resulted in demelanization of conidia (black-head) and fungal hyphae was considered the MDC, as according to and Heleno et al. (2013) and Wan-Mohtar et al. (2017) including the sublethal and subinhibitory (presence of few and much lesser black-head number, respectively) concentration necessary to initiate demelanization in fungus during 72 hours.

3.3.3 Fractional Inhibitory Concentration Indices (FIC) for Combination Study of Antimicrobial properties.

The fractional inhibitory indices (FIC) expresses the interaction of two or more agents in which the concentration of each test agent in combination is expressed as a fraction of the concentration that would produce the same effect when used independently. The FIC was calculated as the MIC of the combination (MIC_{AB}) divided by the MIC of individual component extract, as shown by this formula:

$$FIC_{AB} = \frac{MIC_{AB}}{MIC_{A}} + \frac{MIC_{AB}}{MIC_{B}} \qquad (3,2)$$

The FIC index was calculated as the sum of each component FIC in a combination and interpreted as either synergistic (FIC < 1), additive (FIC = 1), indifferent ($1 < FIC \leq 2$), or antagonistic (FIC > 2) (Fratini et al., 2017).

3.4 Total Phenolic Content and Antioxidant Properties of Single and Combined Vegetable Extracts

3.4.1 Chemicals

Folin-Ciocalteau (F-C) reagent, sodium bicarbonate (Na_2CO_3), ferrous sulfate heptahydrate ($FeSO_4.7H_2O$), ferric chloride hexahydrate ($FeCl_3.6H_2O$), sodium acetate trihydrate ($C_2H_3NaO_2.3H_2O$), and gallic acid ($C_7H_6O_5$) were purchased from R & M Chemicals, UK. Ascorbic acid, 2,4,6-Tri(2-pyridyl)-s-triazine (TPTZ), potassium persulfate ($K_2S_2O_8$), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) were purchased from Sigma, St. Louis. 2, 2-diphenyl-1-picrylhydrazyl (DPPH) was purchased from Sigma, Germany.

3.4.2 Total Phenolic Content (TPC)

Total phenolic content (TPC) of single and combined vegetable extracts was determined using the F-C reagent method with slight modifications (Sulaiman & Ooi, 2012; Wan-Mohtar et al., 2018). In brief, 10 µl of the single and combined vegetable extracts were diluted in distilled water (1 g/ml) and mixed with 25 µl of fresh F-C reagent. After 5 minutes, the solution was mixed with 25 µl of 20% Na_2CO_3 (w/v) solution. Distilled water was added to a final volume of 200 µl, and the solution was incubated in the dark for 30 minutes at room temperature. Absorbance was read at 760 nm using a microplate reader. A standard curve for gallic acid was used to determine the TPC, which was expressed as gallic acid equivalent (mg GAE/g).

3.4.3 Antioxidant Assays

3.4.3.1 Ferric Reducing Antioxidant Power (FRAP) Assay

The ferric reducing antioxidant power (FRAP) assay was modified from a previous report (Benzie & Strain, 1996; Wan-Mohtar et al., 2018). FRAP reagent was freshly prepared (300 mM acetate buffer pH 3.6, 10 mM TPTZ in 40 mM HCl, and 20 mM $FeCl_3.6H_2O$ [10:1:1 ; v/v)), and 20 µl of each single and combined vegetable extracts were diluted in distilled water (1 g/ml) and added with 180 µl of FRAP reagent to the wells of a 96-well plate. The plate was incubated for 30 minutes at room temperature in the dark. Absorbance was measured using a plate reader at 595 nm. Ferrous sulphate ($FeSO_4$) solution was used as a standard, and FRAP activity was calculated as ferrous equivalent (mM FE (II)/mg). Ascorbic acid was used as a positive control.

3.4.3.2 2,2-diphenyl-1-picrylhydrazyl (DPPH) Assay

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay was performed as described previously by Sulaiman & Ooi (2012) and Wan-Mohtar et al. (2018) with slight modifications. A 50 µl aliquot of each vegetable extract (0.31–10.0 mg/ml) was diluted in distilled water and mixed with 150 µl of 0.3 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 517 nm. Antioxidant activity was evaluated over a range of concentrations to establish IC_{50} (the concentration that reduced DPPH absorbance by 50%). Ascorbic acid was used as a positive control.

3.4.3.3 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS)

2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) radical scavenging activity was determined as described previously by Kim et al. (2003) and Wan-Mohtar et al. (2018) with minor modifications. Briefly, 2.45 mM $K_2S_2O_8$ and 7 mM ABTS solutions were prepared and mixed as a reagent before being incubated in the dark at room temperature for 16 h prior to use. The absorbance of the resulting blue-green ABTS radical solution was adjusted to 0.7 ± 0.2 before use. Next, 20 µl of single and combined vegetable extracts (0.25–1.5 g/ml) were mixed with 180 µl of diluted ABTS solution and incubated at room temperature for 20 minutes. Absorbance was read at 734 nm using a microplate reader, with ascorbic acid as a positive reference. The scavenging ability of extracts was expressed as the IC_{50} value, which was the concentration at which 50% of ABTS radicals were scavenged.

3.5 Protective Effect of Single and Combined Vegetable Extracts against Hydroxyl Radical

3.5.1 Toxicity (IC_{50}) Determination of H_2O_2

The toxicity of oxidant solutions towards the viability of NHDF cells was assessed according to the method described by Piah et al. (2010) and Wan-Mohtar et al. (2018). NHDF cells were cultured at a concentration of 3×10^4 cells/ml in a 96-well plate prior to the addition of hydrogen peroxide (H_2O_2). 10 µl of diluted H_2O_2 in DMEM 10% was added into each well. The control wells contained NHDF cells without addition of H_2O_2. The plate was incubated 24 hours, and at the end of the incubation period, viability of cells was assessed by the NRU assay. The concentration of H_2O_2 that can induce 50% cell viability was used for further protective effect assessment.

3.5.2 Determination of Protective Effects

The protective effects of single and combined vegetable extracts against hydroxyl radicals were performed as described previously by Varma et al. (2016) with slight modifications. NHDF cells were seeded at a concentration of 3×10^5 cells/ml in 96-well plates and maintained in DMEM with 10% FBS for 24 hours. Two different experimental sets were performed: (a) pre-treatment of cells with single and combined vegetable extracts for 24 hours prior to exposure to H_2O_2 (100 µM for 1 hour), and (b) post-treatment of cells with single and combined vegetable extracts after H_2O_2 (100 µM for 1 hour) exposure for 24 hours. Ascorbic acid (100 µg/ml) and NHDF cells with no treatment were used as a reference standard. Cells treated with H_2O_2 alone were used as a negative control. After 24 hours of incubation, cell viability was measured using the NRU assay.

3.6 Nitric Oxide (NO) Stimulation Assay

RAW264.7 cells were detached from the culture flask using a cell scraper and centrifuged at 100×g for 10 minutes at 4°C. The cells were then seeded at approximately 5×10^5 cells/mL into a 96-well plate (200 µl of cell suspension in each well) and incubated at 37°C for 24 hours at 5% CO_2. The culture media was replaced with fresh DMEM supplemented with 10% FBS and cells were stimulated with vegetable extracts (25, 100, and 500 µg/ml), with 1 µg/ml of lipopolysaccharide (LPS) from *Escherichia coli* 0111:B4 used as a positive control. Cells were subsequently incubated for 24 hours at 37°C and 5% CO_2. NO production in the culture medium was measured by the Griess reaction using sodium nitrite as the standard (0–100 µM), and 100 µl of Griess reagent (1% sulphanilamide in 5% phosphoric acid and 0.1% N-(1-naphtyl) ethylenediamide dihydrochloride) was mixed with 100 µl of cell supernatant. A microplate reader was

used to measure the absorbance of samples at 540 nm. By using the predetermined sodium nitrite calibration curve, nitrite concentration was expressed as µmol/ml (Varma et al., 2016; Wan-Mohtar et al., 2017).

3.7 Chemical Profiling of Vegetable Extracts (LC-MS/MS Analysis)

3.7.1 UHPLC-QTrap-MS/MS Analysis

The single vegetable extracts (AI-A, CA-A, SG-A, AI-M, and SG-M) was analyzed using an LC/MS-MS system equipped with the 3200 QTrap and UHPLC system (Agilent 1100 Series, California, USA). Injection of 20 µl of samples (5 mg/ml) by an autosampler (Agilent 1100 G1313A, California, USA) onto a C18 column (100 mm × 3µM × 2.0 mm) (Phenomenex Synergi, California USA), eluted with a mobile phase comprising of solvent A (0.1% formic acid with water) and solvent B (0.1% formic acid with acetonitrile). Prior to spectral analysis (negative mode), the chromatographic separation was carried out by the gradient system, 10% of solvent A to 90% of solvent B (0.01 min to 8.0 min), hold for 3 minutes, and returned to original conditions (10% in solvent B) in 1 minute and column re-equilibration for 5 minutes. Identity of the detected compound attained by referring to the molecular ions (*m/z*) with the reference standard of-in house library of the HPLC system was used for identification and also the comparison with previously reported literature.

3.7.2 UHPLC-TWIMS-QTOF-MS/MS Analysis

The major bioactive compounds in *C. asiatica* are the triterpenes, including madecassoside and asiaticoside (Alqahtani et al., 2015; Gray et al., 2018). Thus, CA-M was subjected for a detail identification of the phytochemical compounds by UHPLC-TWIMS-QTOF-MS/MS. The vegetable extract of CA-M (10 µl at 5 mg/ml) was injected onto ACQUITY UPLC I-Class system (Waters Co., Massachusetts, USA), equipped with a binary pump, a vacuum degasser, an autosampler, and a column oven. The chromatographic separation of CA-M was done via ACQUITY UPLC HSS T3 column (100 mm × 2.1 mm × 1.8 µm, Waters Co.), operated at 40°C. A binary gradient system of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in acetonitrile) was used as mobile phase. The separation was performed at a flow rate of 0.6 ml/min, from 1–100% of solvent B (0–18 min) and returned to original conditions 1% of solvent B (20.0 min). Profiling of the compounds was achieved by coupling the HPLC system to an ion mobility mass spectrometer (Vion IMS) QTOF hybrid mass spectrometer (Waters Co.), equipped with electrospray ionization interphase (ESI) in the positive and negative ionization mode. The capillary voltage was adjusted to 1.50 kV, desolvation nitrogen gas (>99.5%) at 120°C with a flow rate of 800 L/h and cone gas flow at 50 L/h. Data were obtained in the range *m/z* 50–1500 Da at 0.1 s/scan. Two independent scans at different collision energies (CE) was acquired in the run, low energy (CE of 4 eV) and high energy (10–40 eV) scan. Argon (99.999%) was used as collision-induced-dissociation (CID) gas. Identity of the detected compound attained by referring to the molecular ions (*m/z*) with the reference standard of in-house library on the AQUITY UPLC I-class instrument and comparison with previous literature.

3.8 Determination of Synergistic Effects of Combined Vegetable Extracts

The potential interactions of the combined vegetable extracts in each assay (proliferation of fibroblast, antioxidative and protective effects, and stimulation of NO) were analysed using the Combination Index (CI) model via CompuSyn software 2.0 (Biosoft, Cambridge, UK). The concentration-response curves of the single and combined vegetable extracts were constructed, and relevant statistics (Combination Index – Fraction affected (CI-Fa) and isobologram) to determine the synergistic/additive/antagonistic interactions were generated. The Combination Index (CI) values denoted synergism (C1 < 1), additive (CI = 1), and antagonism (CI > 1) (Chou, 2006, 2010; Zhou et al., 2017).

3.9 Statistical Analysis

All measurements were carried out in triplicates, and the results were shown as mean ± S.D (standard deviation) using GraphPad Prism software (Version 7.0) (USA). Statistical differences were analysed using one-way or two-way analysis of variance (ANOVA) followed by Tukey's or Dunnet's test.

RESULTS

4.1 Collection of the Vegetables and Yield of the Extracts

Three vegetables namely, *Acalypha indica* L., *Centella asiatica* (L.) Urb., and *Sesbania grandiflora* (L.) Pers. were selected in this study, as shown in Figure 4.1. *A. indica* was collected from Taman OUG, Kuala Lumpur, *C. asiatica* was collected from Rimba Ilmu Botanic Garden, Universiti Malaya, and *S. grandiflora* was collected from Muar, Johor.

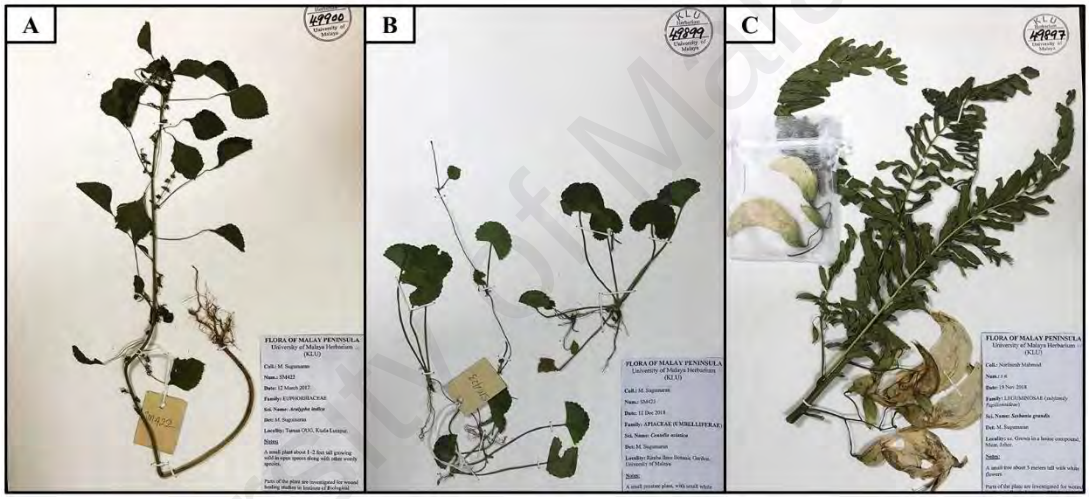


Figure 0.1: (A) *Acalypha indica* L. (B) *Centella asiatica* (L.) Urb. (C) *Sesbania grandiflora* (L.) Pers.

Authentication of the plants and the voucher materials of the specimen were deposited in the herbarium of the Rimba Ilmu Botanical Garden, Institute of Biological Sciences, Universiti Malaya. Table 4.1 summarizes the list of vegetable species, part of the vegetable, voucher numbers, time, and sites of the collection that were used in this study.

Table 0.1: Selected vegetables used in this study.

Vegetable species	Vegetable's part used	Voucher Number	Collection time	Collection site
A. indica	Leaves	KLU 49900	March 2017	Taman OUG, Kuala Lumpur
C. asiatica	Leaves	KLU 49899	December 2018	Rimba Ilmu Botanic Garden, Universiti Malaya
S. grandiflora	Leaves	KLU 49897	November 2018	Muar, Johor

The yield of extracts obtained from the aqueous and methanol extraction was shown in Table 4.2. SG-M produced the highest yield (33.97%) in the methanol extracts. Meanwhile, SG-A produced the highest yield compared to other vegetable species of aqueous extract.

Table 0.2: Methanol and aqueous extract yield (%, w/w) of *A. indica*, *C. asiatica,* and *S. grandiflora*.

Extraction solvent	Yield (%)		
	A. indica **(AI)**	***C. asiatica*** **(CA)**	***S. grandiflora*** **(SG)**
Aqueous (A)	3.43*	2.83*	4.99*
Methanol (M)	10.11#	13.65#	33.97#

* fresh leaves
\# dried leaves

4.2 *In vitro* Wound Healing Assay

4.2.1 NRU Cytotoxicity Assay

As a preliminary experiment, the assessment of toxicity of different concentrations (0–100 µg/ml) of single and combined vegetable extracts against NHDF cells was done by NRU assay. The IC_{50} values of vegetable extracts tested against NHDF are shown in Table 4.3. Any tested compound must be able to sustain more than 75% cell viability of fibroblast cells (Pérez-Díaz et al., 2016) and have high IC_{50} value (> 30 µg/ml) to be considered as non-toxic to the cells (Dante et al., 2019). The highest cytotoxic activity was exhibited by aqueous extract of *S. grandiflora* (SG-A) with IC_{50} 52.61 ± 4.09 µg/ml and the lowest cytotoxicity was shown by the combination of methanol extract of *C. asiatica* and *S. grandiflora* (CA-M+SG-M) with the value of 73.84 ± 1.95 µg/ml. The results showed that no significant cytotoxicity of the treated NHDF when compared to the control (non-treated group).

Table 0.3: Cytotoxicity (IC_{50}) values of single and combined vegetable extracts against normal human dermal fibroblast (NHDF).

Extracts	IC_{50} (µg/ml)*
Control (no treatment)	>100
AI-A	53.36 ± 4.04
CA-A	63.64 ± 3.94
SG-A	52.61 ± 4.09
AI-M	53.64 ± 4.24
CA-M	70.08 ± 2.43
SG-M	72.57 ± 4.24
AI-A+CA-A	73.44 ± 3.75
AI-A+SG-A	58.51 ± 5.23
CA-A+SG-A	67.16 ± 3.83
AI-M+CA-M	61.09 ± 0.92
AI-M+SG-M	67.49 ± 0.42
CA-M+SG-M	73.84 ± 1.95

AI-A = *A. indica* (aqueous extract) AI-M = *A. indica* (methanol extract)
CA-A = *C. asiatica* (aqueous extract) CA-M = *C. asiatica* (methanol extract)
SG-A = *S. grandiflora* (aqueous extract) SG-M = *S. grandiflora* (methanol extract)
*$P < 0.05$ versus control (no treatment)

4.2.2 Proliferation of NHDF Cells

Proliferation of NHDF cells were evaluated at different concentrations of vegetable extracts (25, 50, 75, and 100 µg/ml) via NRU assay. Figure 4.2 and Figure 4.3 showed the percentage of cell viability of single and combined vegetable extracts, respectively. The single and combined vegetable extracts promoted the growth of NHDF cells at 25 µg/ml, and as the concentration of the extract increased, the viability of the cells decreased. Thus, based on the percentage of cell viability, only one concentration of 25 µg/ml was chosen for the scratch assay for migration assessment of NHDF cells. Treatment with 25 µg/ml of the extract increased the proliferation of NHDF cells by 5–25 % compared to the control.

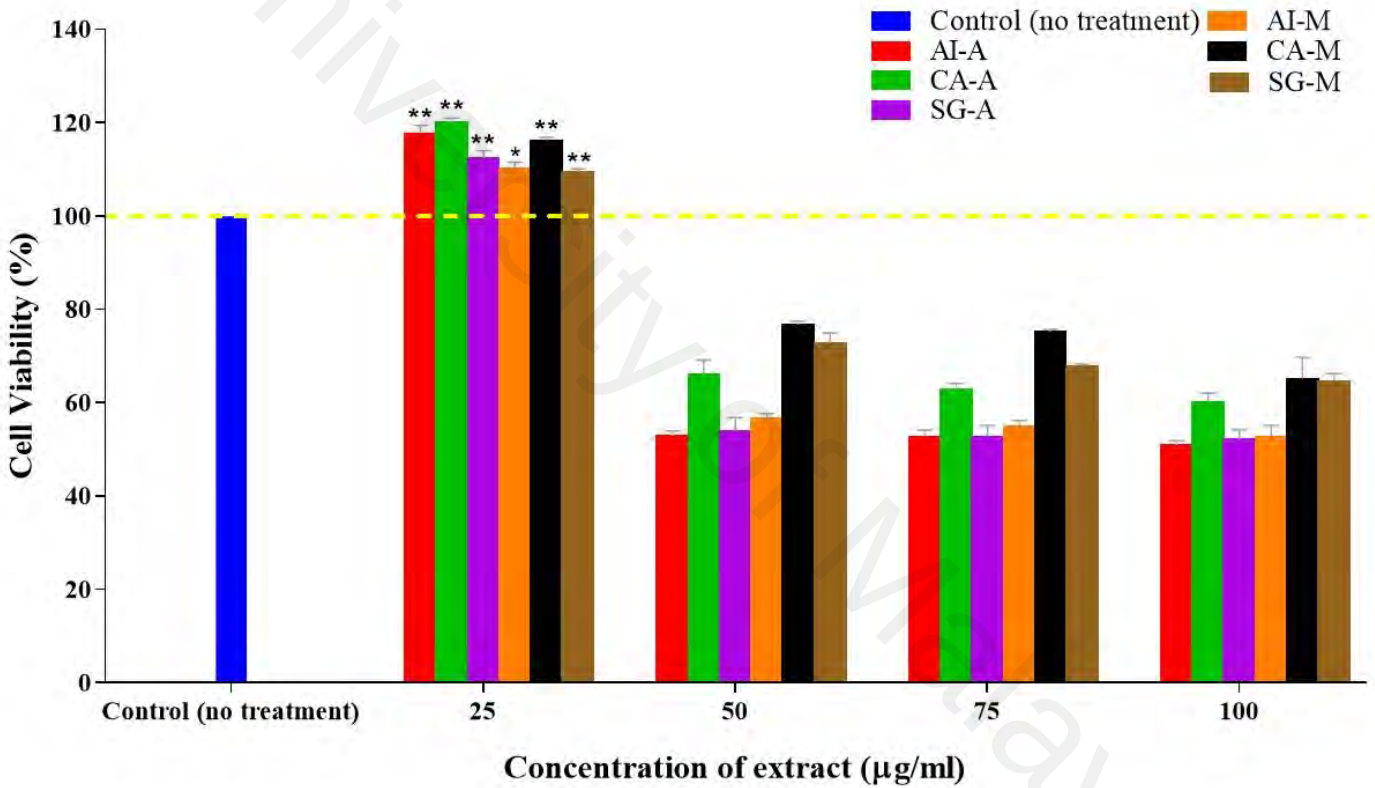


Figure 0.2: Cytotoxicity activity of the single aqueous (AI-A, CA-A, SG-A) and methanol (AI-M, CA-M, SG-M) vegetable extracts against normal human dermal fibroblast (NHDF) cells. Yellow line indicates the viability of cells when treated with media DMEM 1% (control/no treatment). Two-way ANOVA followed by Dunnett's test was used for statistical analysis (*$P < 0.05$ and **$P < 0.0001$).

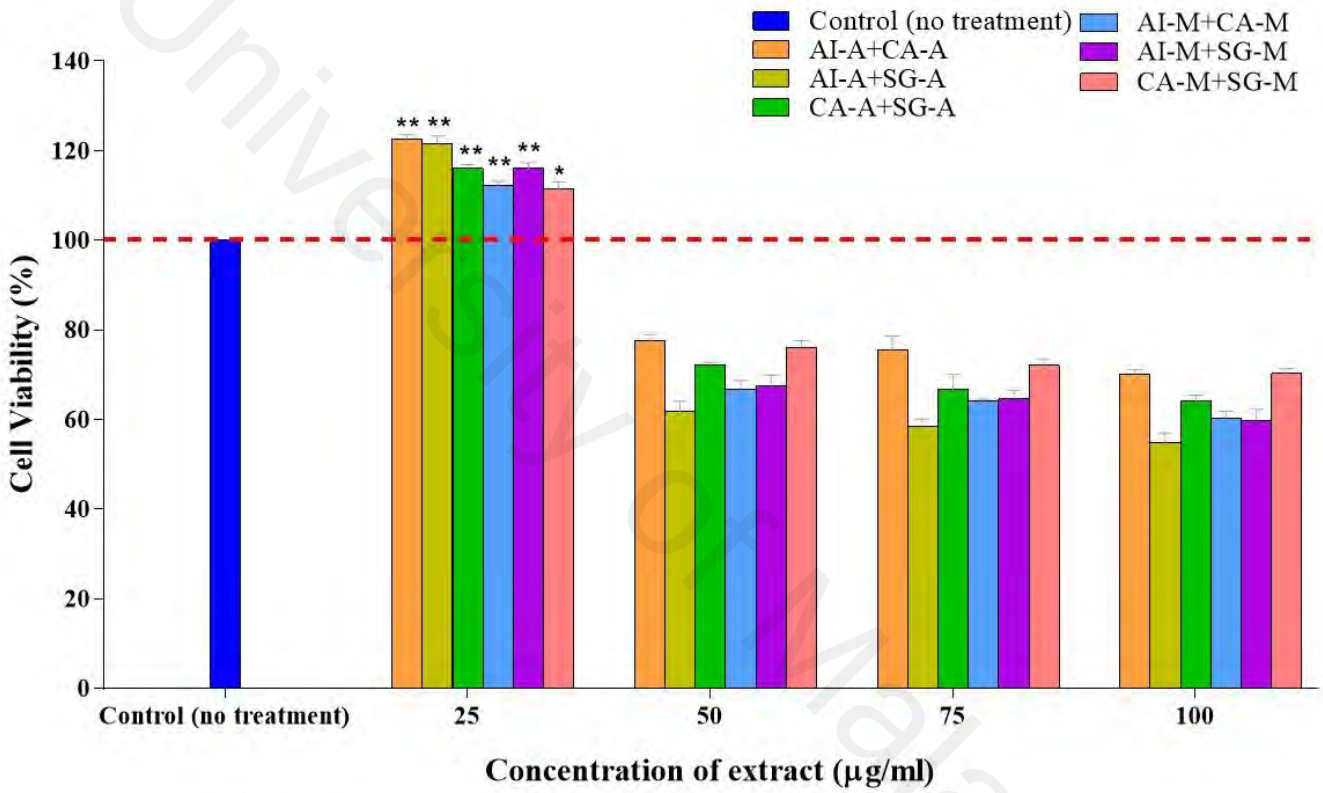


Figure 0.3: Cytotoxicity activity of the single aqueous (AI-A+CA-A, AI-A+SG-A, CA-A+SG-A) and methanol (AI-M+CA-M, AI-M+SG-M, CA-M+SG-M) vegetable extracts against normal human dermal fibroblast (NHDF) cells. Red line indicates the viability of cells when treated with media DMEM 1% (control/no treatment). Two-way ANOVA followed by Dunnett's test was used for statistical analysis (*$P < 0.05$ and **$P < 0.0001$).

4.2.3 Scratch Migration Assay

In this study, the percentage of wound closure in NHDF cells was quantified by an *in vitro* scratch migration assay at 24- and 48-hours post-wounding. The artificial wounds of NHDF were treated with 25 µg/ml of the single (AI-A, CA-A, SG-A, AI-M, CA-M, and SG-M) and combined (AI-A+CA-A, AI-A+SG-A, CA-A+SG-A, AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M) vegetable extracts. As shown in Figure 4.4, migration of NHDF cells into the scratch wound area were observed in the presence single aqueous (AI-A, CA-A, and SG-A) and methanol (AI-M, CA-M, and SG-M) vegetable extracts. Figure 4.5 showed the migration of NHDF cells in the presence of combined aqueous (AI-A+CA-A, AI-A+SG-A, and CA-A+SG-A) and methanol (AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M) vegetable extracts compared to the negative control. Table 4.4 shows the percentage of wound closure at 24 and 48 hours.

Overall, as shown in Figure 4.6, for single extract treatment, the aqueous extract of *C. asiatica* (CA-A) resulted in the highest percentage of wound closure (89.52 ± 4.03%). The rate of fibroblast migration at T48 of the single vegetable extracts in descending order is as follows: CA-A > AI-A > CA-M > AI-M > SG-M > SG-A. For combined extract treatment, the combination of aqueous extract of *A. indica* and *C. asiatica* (AI-A+CA-A) showed the highest percentage of wound closure (90.76 ± 3.62%). The best combination of vegetable extracts to enhance migration of fibroblast in descending order is as follows: AI-A+CA-A > CA-A+SG-A > AI-M+CA-M > AI-A+SG-A > CA-M+SG-M > AI-M+SG-M.

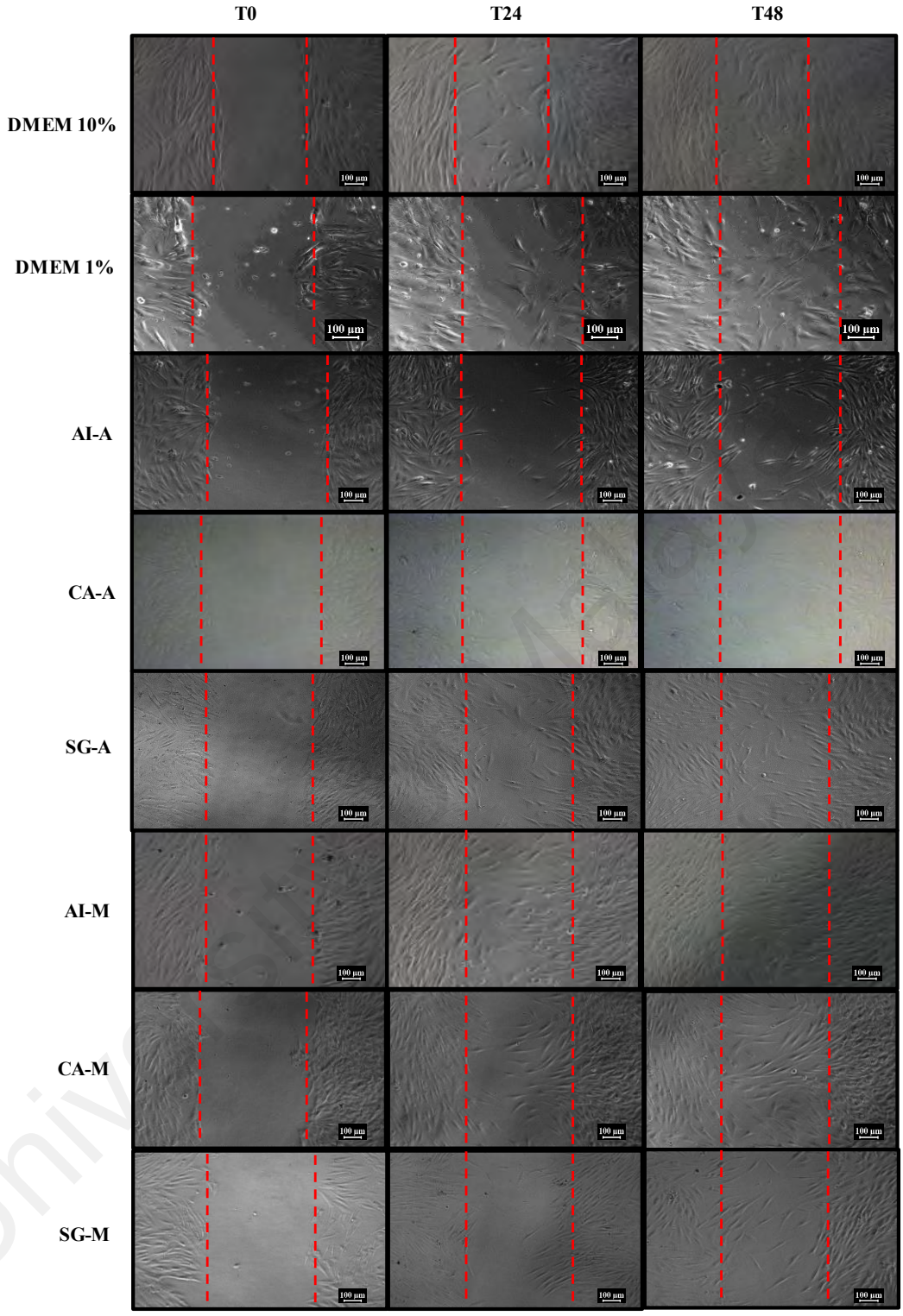


Figure 0.4: Effects of the single aqueous and methanol vegetable extracts on wound closure on NHDF cells. Phase contrast micrographs of dermal fibroblasts cells were taken at 0, 24, and 48 hours after treatment with 25 µg/ml of vegetable extracts. Scale bar 100 µm, 10× magnification (DMEM 10%, AI-A, CA-A, SG-A, AI-M, CA-M, SG-M) and 20× magnification (DMEM 1%). DMEM 10% and DMEM 1% were used as positive and negative control, respectively. The measurement of gap covered by NHDF cells was made after 24 and 48 hours.

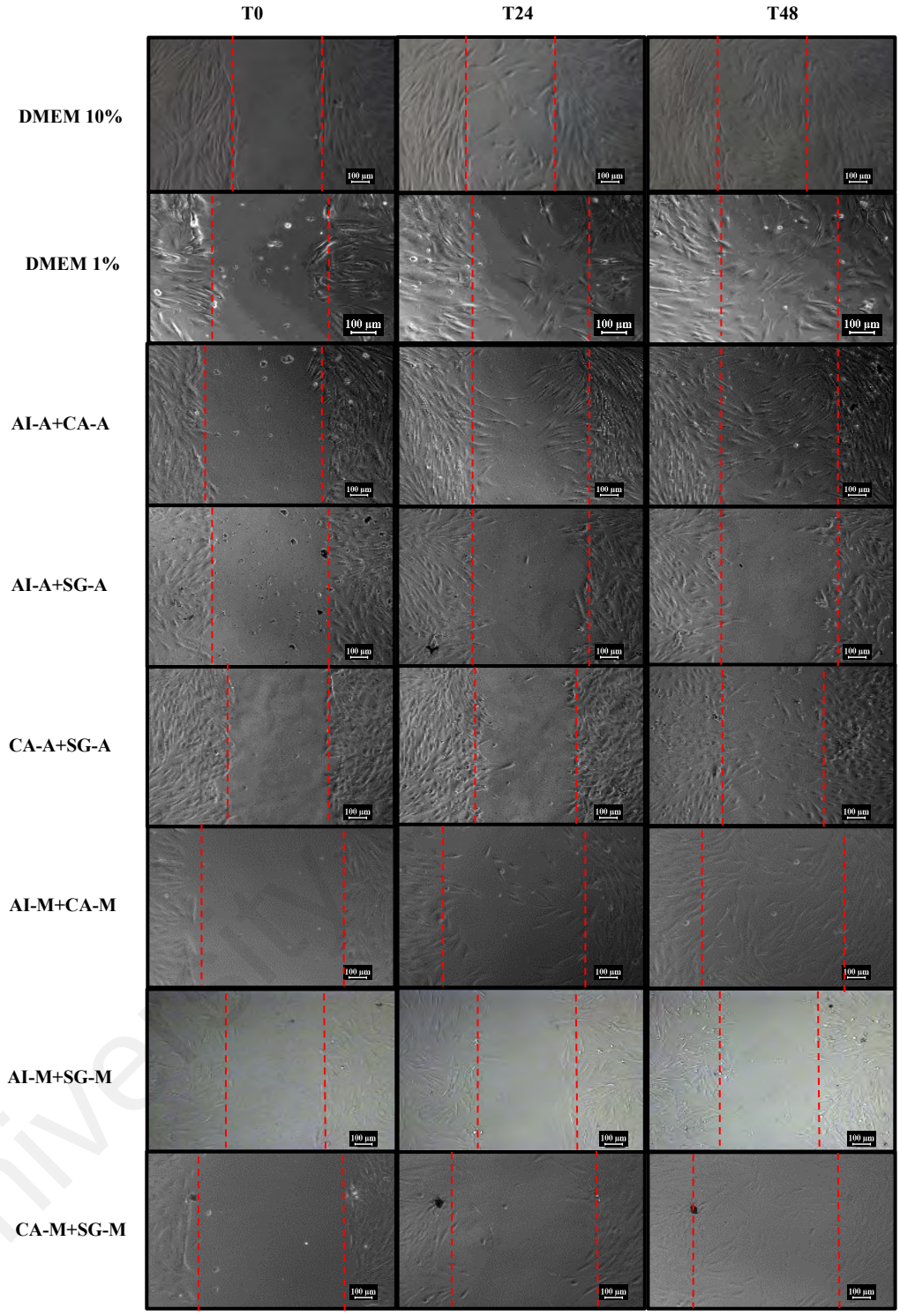


Figure 0.5: Effects of the single aqueous and methanol vegetable extracts on wound closure on NHDF cells. Phase contrast micrographs of dermal fibroblasts cells were taken at 0, 24, and 48 hours after treatment with 25 µg/ml of vegetable extracts. Scale bar 100 µm, 10× magnification (DMEM 10%, AI-A+CA-A, AI-A+SG-A, CA-A+SG-A, AI-M+CA-M, AI-M+SG-M, CA-M+SG-M) and 20× magnification (DMEM 1%). DMEM 10% and DMEM 1% were used as positive and negative control, respectively. The measurement of gap covered by NHDF cells was made after 24 and 48 hours.

Table 0.4: Percentage of wound closure indicated by the migration of NHDF cells into the gap area, at the time interval of 24 and 48 hours.

Extracts / Control	Percentage of wound closure (%)	
	T24	T48
DMEM 10%	64.69 ± 3.47	92.34 ± 2.14
DMEM 1%	45.57 ± 7.79	75.61 ± 1.02
AI-A	63.61 ± 9.37	88.79 ± 3.72[b]
CA-A	59.86 ± 11.05	**89.52 ± 4.03[b]***
SG-A	53.54 ± 4.94	74.67 ± 5.96[ab]
AI-M	54.93 ± 7.81	77.07 ± 4.00[ab]
CA-M	60.87 ± 6.67	77.57 ± 3.17[ab]
SG-M	50.72 ± 12.48	75.12 ± 8.07[ab]
AI-A+CA-A	69.63 ± 0.26	**90.76 ± 3.62[b]***
AI-A+SG-A	55.28 ± 5.96	73.14 ± 6.12[ab]
CA-A+SG-A	51.98 ± 12.65	76.70 ± 3.92[ab]
AI-M+CA-M	60.84 ± 17.84	73.32 ± 3.19[ab]
AI-M+SG-M	54.71 ± 12.42	67.93 ± 3.84[a]
CA-M+SG-M	54.28 ± 2.79	69.47 ± 2.53[a]

AI-A = *A. indica* aqueous extract
CA-A = *C. asiatica* aqueous extract
SG-A = *S. grandiflora* aqueous extract
AI-M = *A. indica* methanol extract
CA-M = *C. asiatica* methanol extract
SG-M = *S. grandiflora* methanol extract

DMEM 10% and DMEM 1% were used as positive and negative control, respectively. The data were expressed as mean ± S.D. of triplicate values.

One-way ANOVA was used for statistical analysis. **P* < 0.05 versus negative control (DMEM 1%) and [a,b] mean values in a column indicates significance differences at *P* < 0.05.

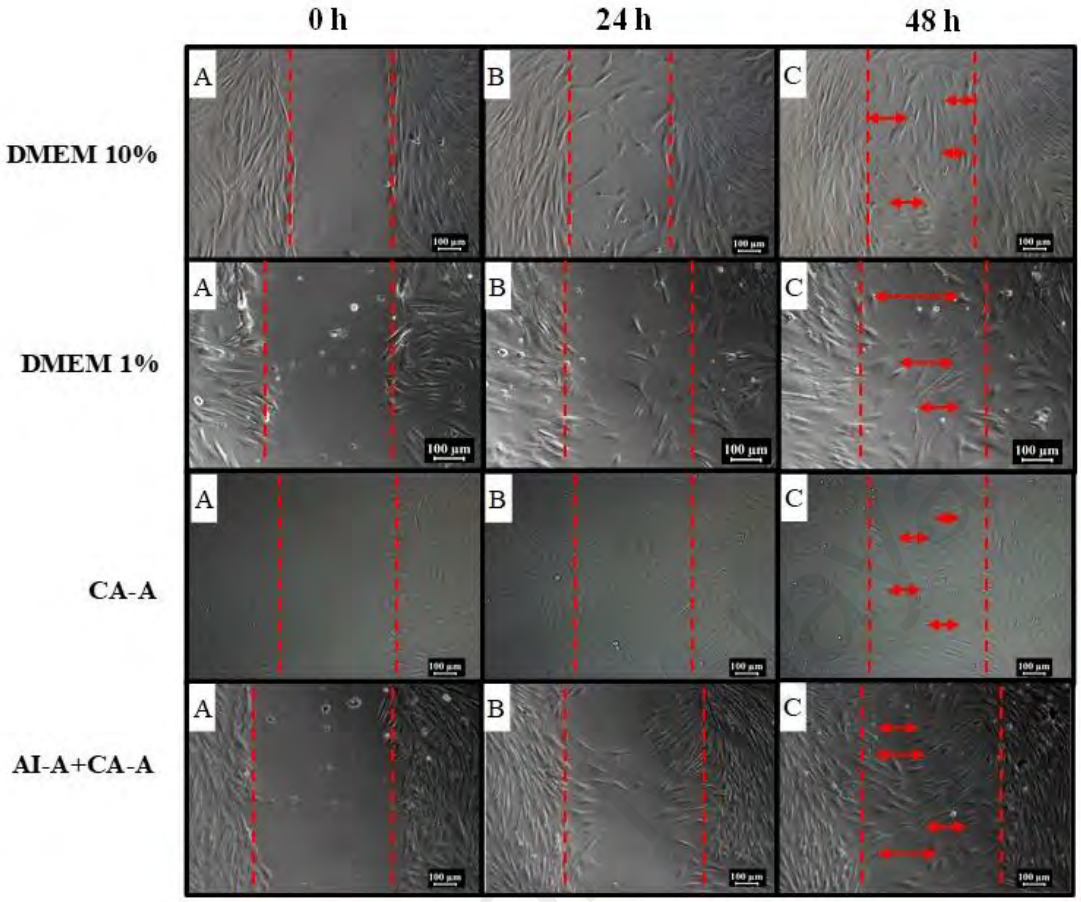


Figure 0.6: Migration of NHDF cells treated with 25 µg/ml of single and combined aqueous vegetable extracts. Phase contrast micrographs of NHDF cells were taken at 0 (A), 24 (B), and 48 hours (C). DMEM 10% served as the positive control, while DMEM 1% served as the negative control. Single extract of CA-A and combined extract of AI-A+CA-A showed the highest percentage of fibroblast migration (red arrow) compared to other vegetable extracts, 89.52 ± 4.03% and 90.76 ± 3.62% respectively. Scale bar at 100 µm, 10× magnification (DMEM 10%, CA-A, AI-A+CA-A), 20× magnification (DMEM 1%).

4.2.4 Synergism of Combined Vegetable Extracts on Fibroblast Proliferation

Table 4.5 showed the CI values for each combination of vegetable extracts, executed by the CompuSyn software. The Combination Index-Fraction affected (CI-Fa) curves in Figure 4.7 suggested that only AI-A+CA-A have synergistic interaction (CI <1) at the tested concentration (25 µg/ml). Also, high Fa value (Fa = 0.9076) indicated that AI-A+CA-A is appropriate for the proliferation of fibroblast.

Table 0.5: CI values and interactions of the combined vegetable extracts.

Combination	Total dose (µg/ml)	Fa[#]	CI*	Interaction
AI-A+CA-A	**25**	**0.9076**	**0.8135**	**Synergism**
AI-A+SG-A	25	0.7314	2.3091	Antagonism
CA-A+SG-A	25	0.7670	1.9419	Antagonism
AI-M+CA-M	25	0.7332	1.3363	Antagonism
AI-M+SG-M	25	0.6793	1.7447	Antagonism
CA-M+SG-M	25	0.6947	1.6136	Antagonism

AI-A = *A. indica* aqueous extract
CA-A = *C. asiatica* aqueous extract
SG-A = *S. grandiflora* aqueous extract
#Fa = Fractional affected
AI-M = *A. indica* methanol extract
CA-M = *C. asiatica* methanol extract
SG-M = *S. grandiflora* methanol extract
*CI = Combination Index

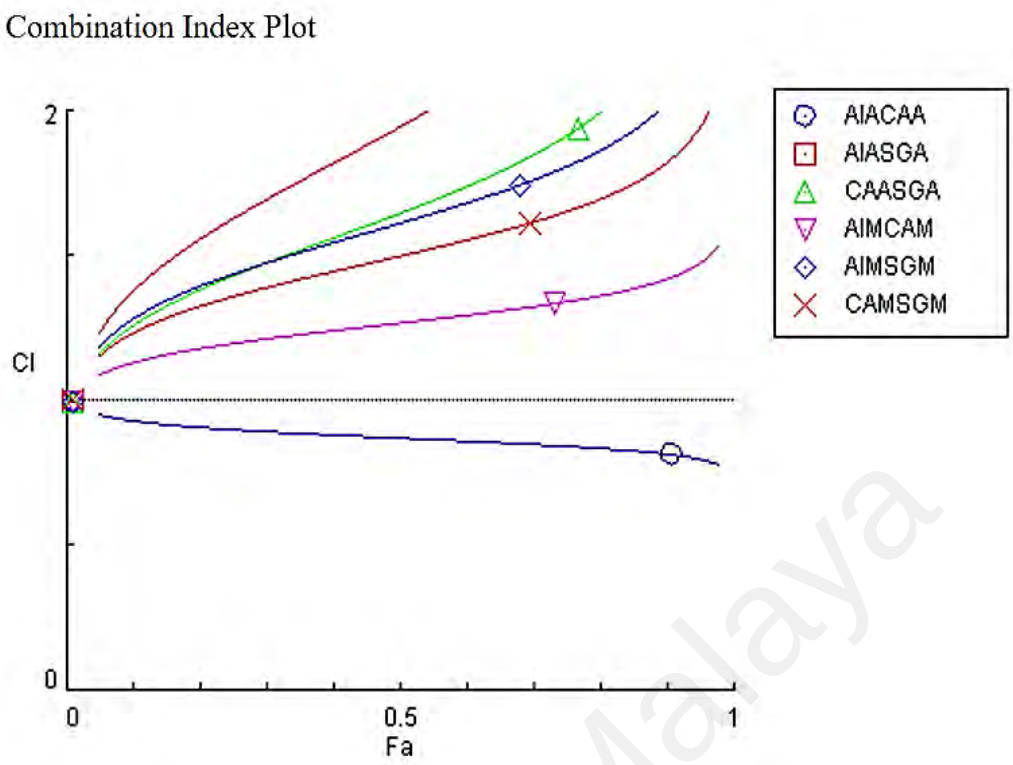


Figure 0.7: Synergism analysis of all combined vegetable extracts (1:1) on the enhancement of fibroblast proliferation and migration. CI values were obtained via CompuSyn software where synergism at CI < 1, additive at CI = 0, and antagonism at CI > 1.

4.3 Antimicrobial Activity

4.3.1 Antibacterial Activity

4.3.1.1 Kirby Disc Diffusion Assay

The antibacterial effects of the single and combined vegetable extracts were tested against seven species of bacteria. The sensitivity of the vegetable extracts was measure quantitatively and qualitatively by the presence or absence of inhibition zones and zone diameters. The findings of these tests are summarized in Table 4.6 (zone diameters) and Figure 4.8 (graphical analysis), respectively.

At 800 mg/ml (dose-dependent reaction), AI-A against *E. coli* (12.0 ± 0.4 mm) and *M. luteus* (13.9 ± 0.6 mm), CA-M against *B. cereus* (10.3 ± 1.2 mm)**,** SG-M against *S. marcescens* (13.0 ± 0.5 mm) and *S. aureus* (9.7 ± 0.1 mm), AI-M+CA-M against *B. subtilis* (10.5 ± 0.4 mm), and CA-M+SG-M against *S. epidermidis* (8.5 ± 0.4 mm) demonstrated significant and maximum inhibition zone diameters compared to the negative control (sterile disc 6 mm). Vancomycin (30 µg) was used as positive control and was effective against all tested bacteria except for *S. marcescens*, which is vancomycin resistant. Interestingly, AI-A, AI-M, CA-M, SG-M, AI-A+CA-A, AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M produced inhibition zones at 400–800 mg/ml (6.7–13.0 mm) against *S. marcescens*. Overall, the results demonstrated that methanol extracts exhibited better antibacterial activities compared to the aqueous vegetable extracts.

Table 0.6: Antibacterial activities of single and combined vegetable extracts using disc diffusion assay.

No. / G	Bacteria	Extracts	Concentration of extracts: Diameter zone of inhibition[a,b] (mm) 200 mg/ml	400 mg/ml	600 mg/ml	800 mg/ml	VANC[c] (30 µg)	Sterile disc[c]
1 (G-)	*Escherichia coli*	**AI-A**	**9.0 ± 0.5***	**9.9 ± 0.9***	**10.9 ± 0.9***	**12.0 ± 0.4***	13.5 ± 0.3	6.1 ± 0.1
		AI-M	NZ	6.5 ± 0.3	6.8 ± 0.1	7.5 ± 0.2*		
		CA-M	NZ	6.4 ± 0.1	6.6 ± 0.1	6.9 ± 0.2*		
		SG-M	6.8 ± 0.4	7.5 ± 0.3*	8.9 ± 0.5*	10.0 ± 0.5*		
		AI-A+CA-A	7.3 ± 0.6*	7.8 ± 0.4*	8.2 ± 0.4*	8.9 ± 0.4*		
		AI-A+SG-A	6.7 ± 0.1	8.2 ± 0.5*	8.3 ± 0.3*	9.3 ± 0.3*		
		AI-M+CA-M	NZ	NZ	7.3 ± 0.5*	8.1 ± 0.2*		
		AI-M+SG-M	7.0 ± 0.4*	7.9 ± 0.6*	9.0 ± 0.5*	10.0 ± 0.3*		
		CA-M+SG-M	6.7 ± 0.3	7.5 ± 0.2*	8.4 ± 0.4*	9.2 ± 0.4*		
2 (G-)	*Serratia marcescens*	AI-A	8.9 ± 0.9*	9.7 ± 0.9*	11.1 ± 0.8*	12.2 ± 0.7*	6.0 ± 0.0	6.2 ± 0.2
		AI-M	6.6 + 0.1	7.4 ± 0.1*	7.8 ±0.3*	9.1 ± 0.4*		
		CA-M	NZ	6.7 ± 0.1	7.4 + 0.2*	12.5 ± 0.8*		
		SG-M	**6.8 ± 0.1***	**10.8 ± 0.4***	**11.7 ± 0.3***	**13.0 ± 0.5***		
		AI-A+CA-A	6.7 ± 0.3	7.3 ± 0.1*	7.9 ± 0.2*	8.4 ± 0.1*		
		AI-M+CA-M	NZ	6.7 ± 0.3	7.2 ± 0.1*	8.1 ± 0.2*		
		AI-M+SG-M	NZ	6.8 ± 0.4	7.4 ± 0.1*	8.8 ± 0.5*		
		CA-M+SG-M	NZ	7.3 ± 0.6*	8.5 ± 0.9*	8.9 ± 0.1*		

Table 4.6, continued

3 (G+)	*Bacillus subtilis*	AI-M	NZ	6.8 ± 0.1	8.1 ± 0.5*	9.2 ± 0.4*	16.2 ± 0.4	6.2 ± 0.1
		CA-M	NZ	NZ	6.6 ± 0.1	8.0 ± 0.2*		
		SG-M	**6.7 ± 0.2**	**8.8 ± 0.1***	**9.5 ± 0.2***	**11.8 ± 0.4***		
		AI-M+CA-M	NZ	6.5 ± 0.1	7.6 ± 0.6*	10.5 ± 0.4*		
		AI-M+SG-M	NZ	NZ	6.7 ± 0.3	7.4 ± 0.4*		
		CA-M+SG-M	NZ	NZ	6.9 ± 0.3*	8.0 ± 0.1*		
4 (G+)	*Bacillus cereus*	CA-A	NZ	6.6 ± 0.1	7.6 ± 0.4*	7.9 ± 0.6*	15.0 ± 0.2	6.1 ± 0.1
		AI-M	6.6 ± 0.1	7.7 ± 0.3*	8.3 ± 0.3*	9.9 ± 0.4*		
		CA-M	**6.4 ± 0.3**	**7.4 ± 0.8***	**8.6 ± 1.0***	**10.3 ± 1.2***		
		SG-M	7.5 ± 0.9*	8.3 ± 0.9*	9.1 ± 0.4*	10.1 ± 0.4*		
		AI-A+CA-A	7.1 ± 0.2*	7.6 ± 0.2*	8.3 ± 0.3*	8.7 ± 0.3*		
		AI-A+SG-A	7.0 ± 0.1*	7.3 ± 0.1*	7.7 ± 0.2*	8.4 ± 0.1*		
		AI-M+CA-M	6.6 ± 0.1	6.9 ± 0.1*	8.7 ± 0.3*	9.9 ± 0.5*		
		AI-M+SG-M	7.1 ± 0.9*	7.5 ± 1.0*	9.0 ± 2.4*	9.6 ± 2.5*		
5 (G+)	*Staphylococcus aureus*	AI-M	NZ	NZ	6.6 ± 0.1	7.2 ± 0.1*	12.4 ± 0.6	6.2 ± 0.1
		CA-M	NZ	NZ	6.4 ± 0.1	6.6 ± 0.1*		
		SG-M	**7.2 ± 0.2***	**7.4 ± 0.3***	**8.6 ± 0.4***	**9.7 ± 0.1***		
		AI-M+CA-M	6.7 ± 0.2	7.0 ± 0.2*	7.3 ± 0.4**	8.0 ± 0.5*		
		AI-M+SG-M	6.6 ± 0.2	6.8 ± 0.1	7.1 ± 0.3*	7.7 ± 0.2*		

Table 4.6, continued

6 (G+)	*Staphylococcus epidermidis*	SG-M	6.5 ± 0.1	7.7 ± 0.2*	8.0 ± 0.1*	8.4 ± 0.1*		
		AI-A+CA-A	6.6 ± 0.1	7.1 ± 0.2*	8.0 ± 0.3*	8.4 ± 0.1*		
		AI-M+CA-M	NZ	6.6 ± 0.1	7.1 ± 0.5*	7.8 ± 0.6*	13.4 ± 0.4	6.1 ± 0.1
		AI-M+SG-M	NZ	NZ	6.9 ± 0.2*	7.7 ± 0.1*		
		CA-M+SG-M	**NZ**	**6.8 ± 0.1**	**7.7 ± 0.1***	**8.5 ± 0.4***		
7 (G+)	*Micrococcus luteus*	**AI-A**	**7.2 ± 0.4***	**9.0 ± 0.4***	**11.0 ± 0.4***	**13.9 ± 0.6***		
		CA-A	6.7 ± 0.4	7.7 ± 0.3*	8.2 ± 0.4*	8.6 ± 0.4*		
		AI-M	6.9 ± 0.2*	9.0 ± 0.8*	9.2 ± 0.6*	11.9 ± 0.6*		
		CA-M	NZ	6.7 ± 0.2	7.6 ± 0.4*	9.4 ± 0.1*		
		SG-M	NZ	6.9 ± 0.3*	7.6 ± 0.4*	9.4 ± 0.1*		
		AI-A+CA-A	6.7 ± 0.2	7.3 ± 0.4*	8.1 ± 0.2*	9.5 ± 0.4*	12.7 ± 0.2	6.2 ± 0.1
		AI-A+SG-A	NZ	NZ	6.8 ± 0.1	8.1 ± 0.3*		
		CA-A+SG-A	NZ	NZ	6.7 ± 0.2	7.5 ± 0.2*		
		AI-M+CA-M	NZ	6.7 ± 0.1	8.1 ± 0.3*	8.8 ± 0.4*		
		AI-M+SG-M	6.5 ± 0.1	7.1 ± 0.2*	7.6 ± 0.4*	8.4 ± 0.6*		
		CA-M+SG-M	NZ	6.5 ± 0.3	7.4 ± 0.2*	7.8 ± 0.1*		

**NZ= No Zone

[a] Values represent the mean ± SD, n=3

[b] Sterile disc size used is 6 mm, positive antimicrobial activities were more than 6 mm.

[c] Vancomycin (VANC) and empty sterile disc were used as positive and negative control, respectively.

Two-way ANOVA followed by Dunnett's test was used for statistical analysis and *$P < 0.05$ versus negative control (sterile disc 6 mm).

AI-A= *A. indica* aqueous extract AI-M = *A. indica* methanol extract

CA-A = *C. asiatica* aqueous extract CA-M = *C. asiatica* methanol extract

SG-A= *S. grandiflora* aqueous extract SG-M = *S. grandiflora* methanol extract

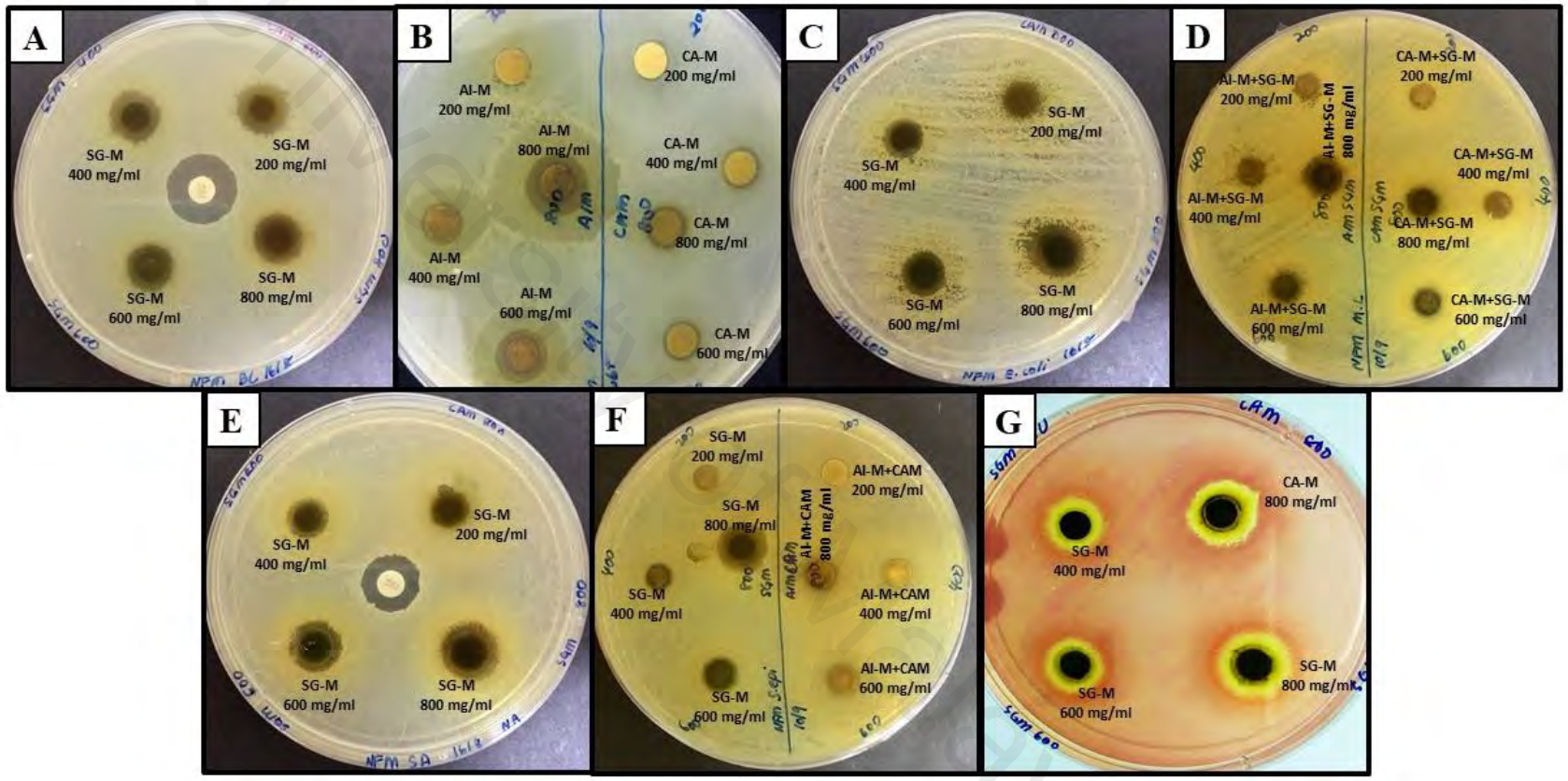


Figure 0.8: Antibacterial activities of single and combined vegetable extracts at various concentration (200, 400, 600, and 800 mg/ml) shown on plate cultures. A) *B. cereus*, B) *B. subtilis*, C) *E. coli*, D) *M. luteus*, E) *S. aureus*, F) *S. epidermidis*, and G) *S. Marcescens*.

4.3.2 Antifungal-demelanizing Activity

4.3.2.1 Antifungal Activity

The antifungal activity of the single and combined vegetable extracts is shown in Table 4.7, and only methanol extracts (single and combined) exhibited antifungal properties. AI-M showed the preeminent antifungal activity compared to others with MIC of 50 mg/ml and MFC of 100 mg/ml. Single extracts of CA-M and SG-M and combination extracts of AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M have similar MIC of 100 mg/ml and MFC of 200 mg/ml. MIC for all single and combined aqueous vegetable extracts was greater than 300 mg/ml.

Table 0.7: Antifungal activity, minimum inhibitory concentration (MIC), and minimum fungicidal concentration (MFC) of single and combined vegetable extracts (mg/ml).

Extracts	*Aspergillus niger*	
	MIC (mg/ml)	MFC (mg/ml)
AI-A	>300	NA*
CA-A	>300	NA
SG-A	>300	NA
AI-M	50	100
CA-M	100	200
SG-M	100	200
AI-A+CA-A	>300	NA
AI-A+SG-A	>300	NA
CA-A+SG-A	>300	NA
AI-M+CA-M	100	200
AI-M+SG-M	100	200
CA-M+SG-M	100	200
Fluconazole (positive control)	1	2
DMSO 5% (negative control)	NA	NA

*NA = not available
AI-A = *A. indica* aqueous extract
CA-A = *C. asiatica* aqueous extract
SG-A = *S. grandiflora* aqueous extract
AI-M = *A. indica* methanol extract
CA-M = *C. asiatica* methanol extract
SG-M = *S. grandiflora* methanol extract

4.3.2.2 Demelanizing Activity

The demelanizing activity of the single (Figure 4.9) and combined (Figure 4.10) vegetable methanol extracts were tested against *A. niger*. The tested concentrations for both single and combined vegetable extracts were at 6.25, 12.5, 25, 50, 100, and 200 mg/ml and the results showed that the demelanizing activity was in a dose-dependent manner. More black heads (conidiophores) was observed as the concentration of the vegetable extracts decreased, indicating weaker demelanizing reaction. The results were reported as minimum demelanizing concentrations (MDCs), that defined as sublethal and subinhibitory concentration required to cause demelanization of fungus in 72 hours. In this experiment, antifungal-demelanizing activity showed only in the single and combined methanol vegetable extracts, respectively. Whereas, no activity for all aqueous single and combined vegetable extracts, as shown in Figure 4.11.

For single extract (Figure 4.9), the subinhibitory concentration for AI-M was 25 mg/ml, whereas the sublethal concentration was 50 mg/ml. Meanwhile, the combined extract (Figure 4.10) showed the same subinhibitory concentration (50 mg/ml) and sublethal concentration (100 mg/ml) for CA-M, SG-M, AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M. Significant morphological changes and depigmentation of the melanin (heads) of *A. niger* are shown in Figure 4.12. Demelanized culture of tested fungi displayed a remarkable small number of heads (Figure 4.12 A and 4.12 B) compared to the untreated culture (Figure 4.12 C). The demelanization and reduction of heads of *A. niger* were observed under a light microscope (Figure 4.12 D–F).

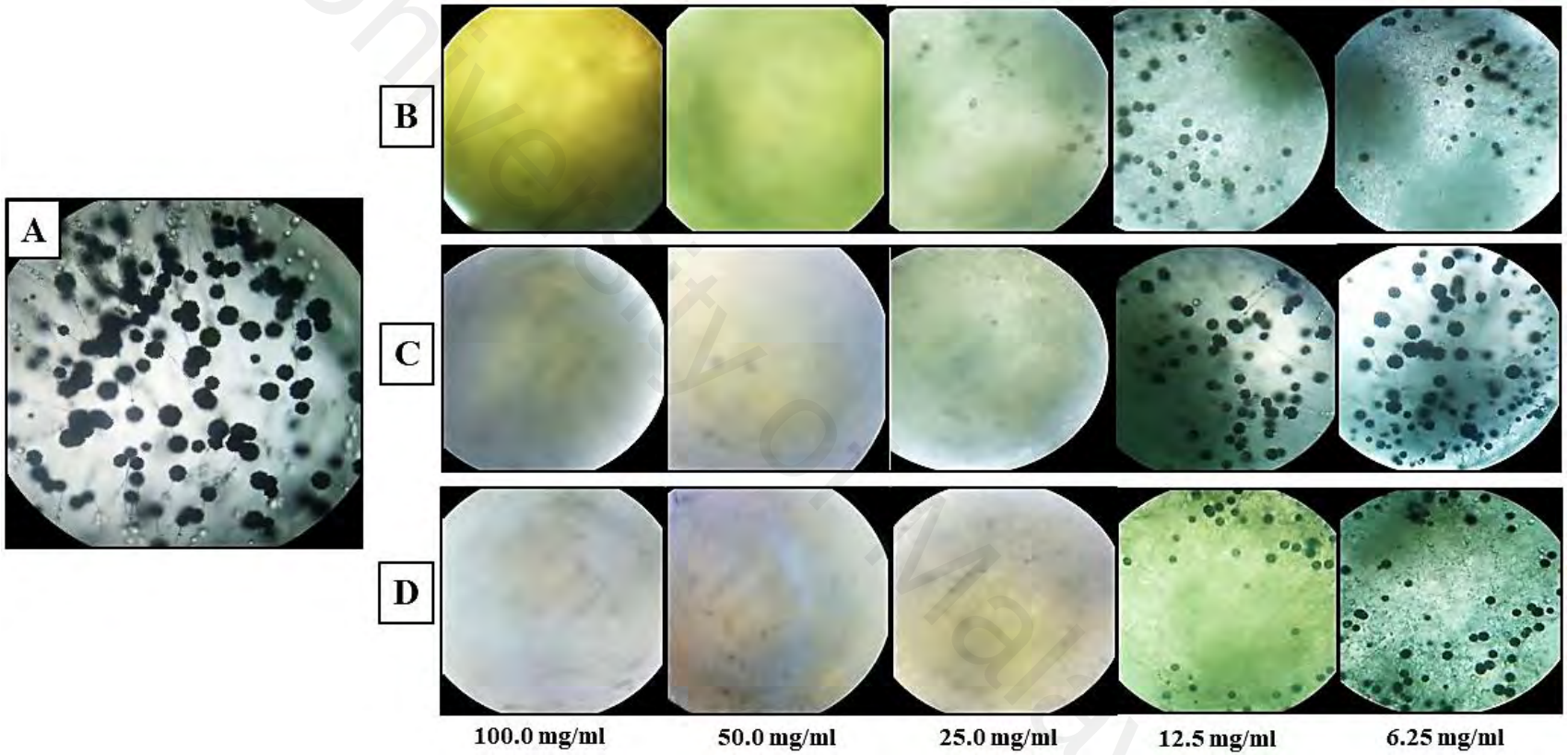


Figure 0.9: Demelanizing properties for single vegetable extracts at concentration 6.25–100 mg/ml on *A. niger*. (A) Culture without any treatment as control; (B) Culture treated with AI-M; (C) Culture treated with CA-M; and (D) Culture treated with SG-M. *Images were obtained under light microscope.

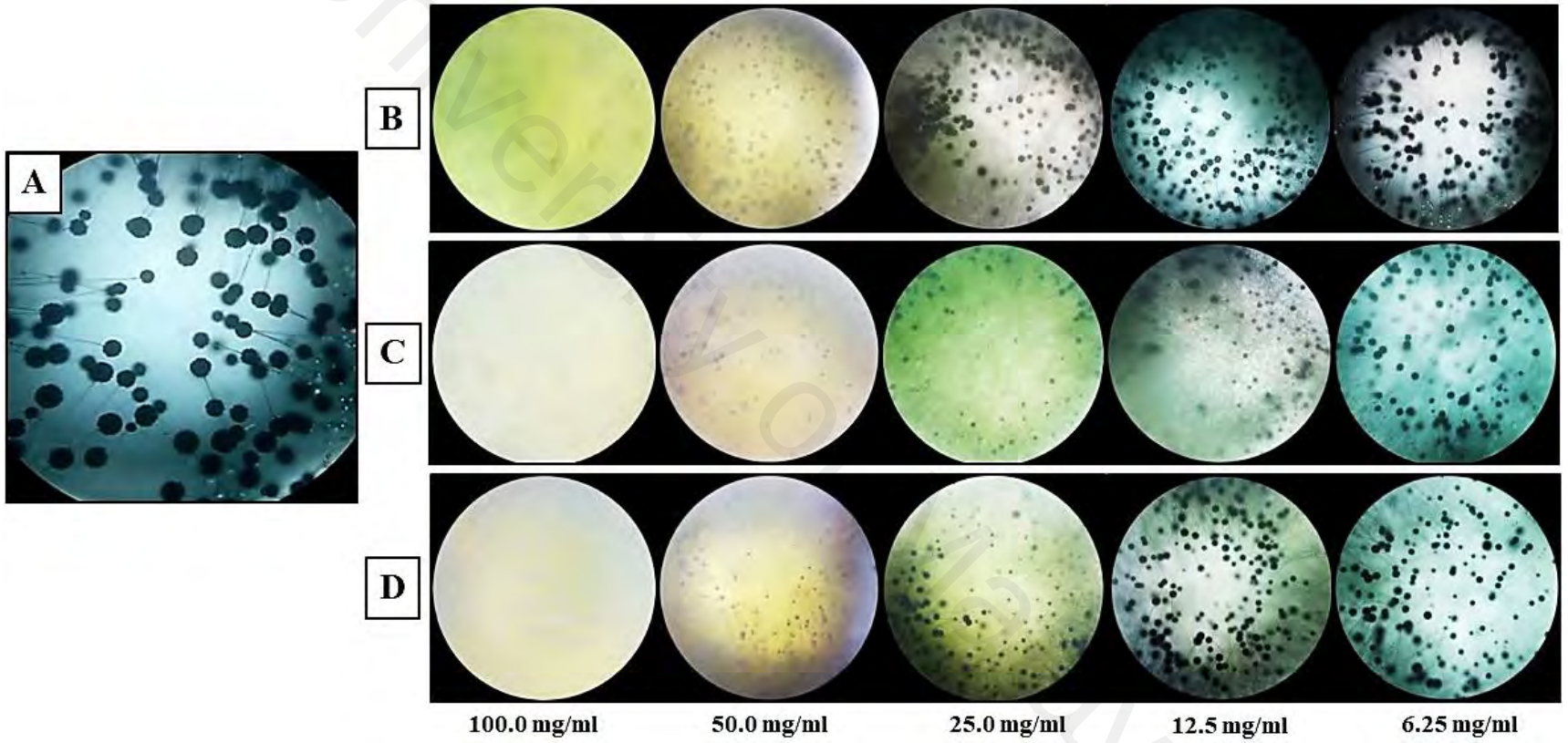


Figure 0.10: Demelanizing properties of combined vegetable extracts at concentration 6.25–100 mg/ml on *A. niger*. (A) Culture without any treatment as control; (B) Culture treated with AI-M+CA-M; (C) Culture treated with AI-M+SG-M; and (D) Culture treated with CA-M+SG-M. *Images were obtained under light microscope.

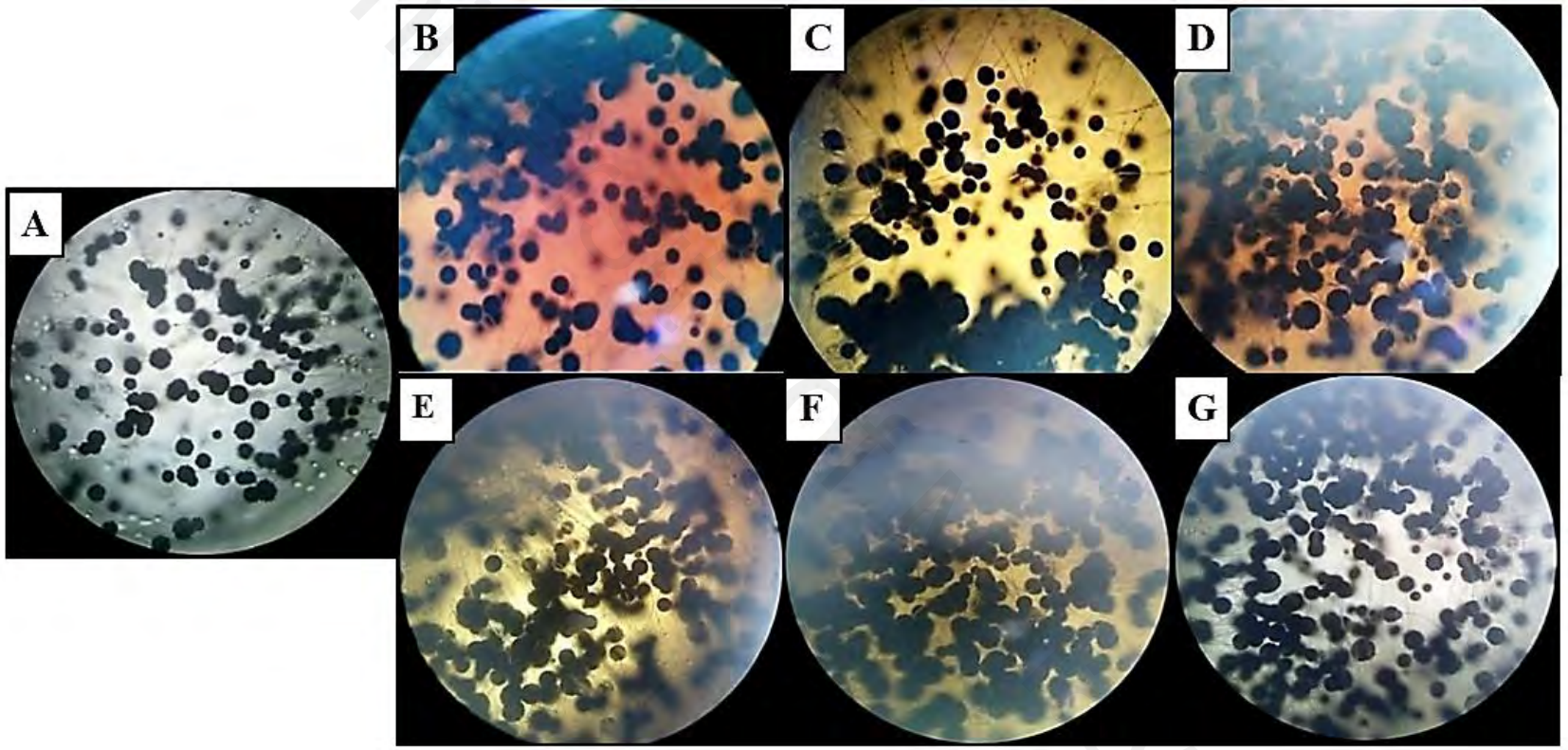


Figure 0.11: Demelanizing properties for single and combined vegetable aqueous extracts at concentration 100 mg/ml on *A. niger*. (A) Culture without any treatment as control; (B) Culture treated with AI-A; (C) Culture treated with CA-A; and (D) Culture treated with SG-A; (E) Culture treated with AI-A+CA-A; (F) Culture treated with AI-A+SG-A; and (G) Culture treated with CA-A+SG-A. *Images were obtained under light microscope.

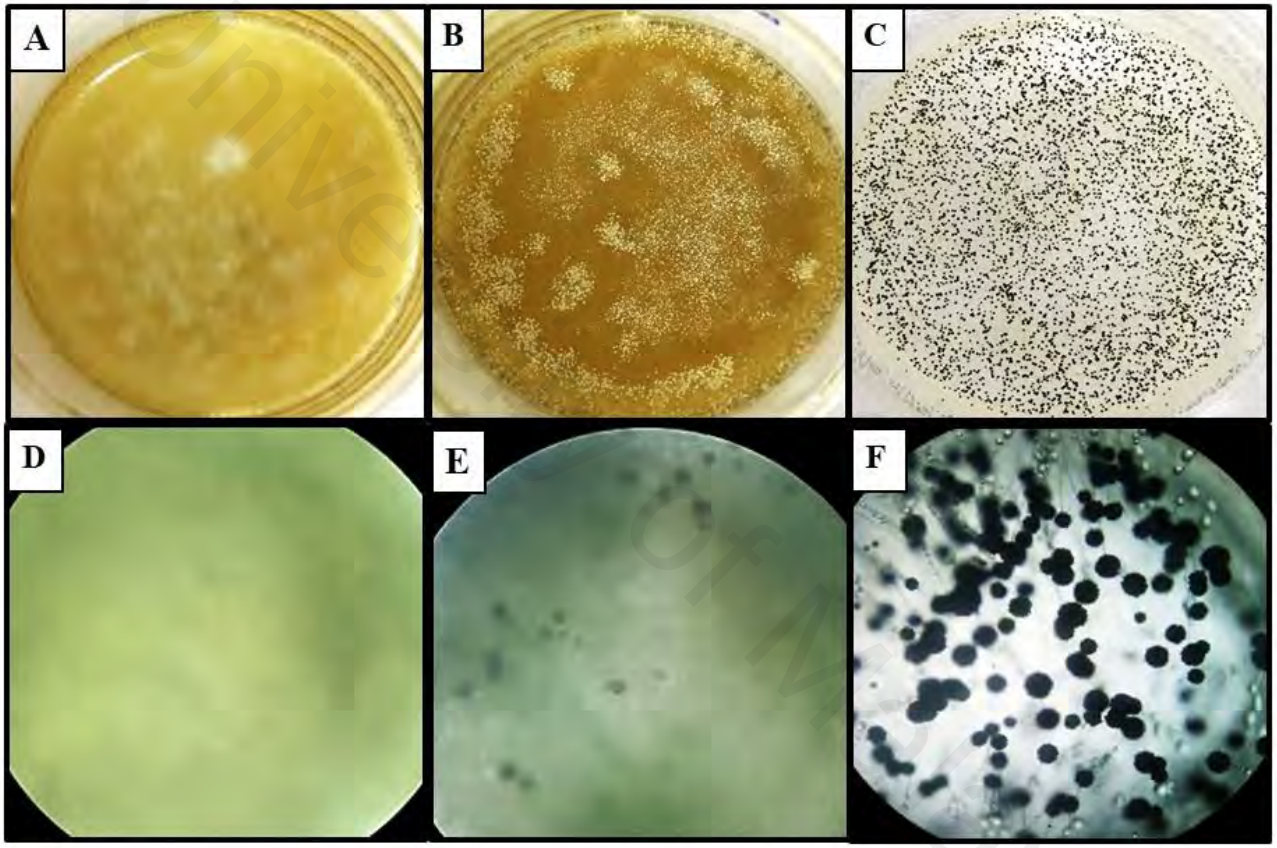


Figure 0.12: Antifungal-demelanizing activity of different concentration of methanol extract of *A. indica* (AI-M). (A) Demelanized mycelium of *A. niger* at 50 mg/ml of AI-M; (B) Mycelium of *A. niger* treated with AI-M at 25 mg/ml; (C) Normal mycelium of *A. niger* without any treatment; (D) Culture of *A. niger* with few number of heads treated with AI-M 100 mg/ml; (E) Culture of *A. niger* with small number of heads treated with 12.5 mg/ml of AI-M; (F) Typical culture of *A. niger* with numerous heads; (D-F) Images were obtained under light microscope. *Lesser black heads (conidiophores) indicates stronger demelanizing properties.

4.3.3 Fractional Inhibitory Concentration (FIC) Indices of Antimicrobial Activities

Table 4.6 showed that antibacterial activities were exhibited at higher concentration of extract (≥ 200 mg/ml). Only methanol vegetable extracts exhibited antifungal activities, and Table 4.8 shows the FIC values and interactions of the combined vegetable extracts.

Table 0.8: Interaction of the combined vegetable extracts.

Combination	FIC	Interaction
AI-A + CA-A	NA*	NA
AI-A + SG-A	NA	NA
CA-A + SG-A	NA	NA
AI-M+CA-M	3	Antagonistic
AI-M+SG-M	3	Antagonistic
CA-M+SG-M	2	Antagonistic

*NA = Not Available
AI-M = *A. indica* methanol extract
CA-M = *C. asiatica* methanol extract
SG-M = *S. grandiflora* methanol extract

4.4 Protective Effects Against Hydroxyl Radical

4.4.1 Toxicity (IC_{50}) Determination of H_2O_2

Determination of the IC_{50} of hydrogen peroxide (H_2O_2) against NHDF cells was performed using NRU assay, and the quantification is based on the absorption of neutral red dye into lysozyme of living cells. The percentage of cell viability is directly proportional to the red dye intensity. Figure 4.13 shows that the highest cytotoxicity at a concentration H_2O_2 of 100000 µM, in which only 6.8% of cells were viable. This preliminary test is important to find the concentration of H_2O_2 that can induce 50% of cell viability, and at concentration 100 µM, the percentage of viable cells was 45.7%. Because of that, the concentration of 100 µM was chosen for further protectivity (pre-treatment and post-treatment) test.

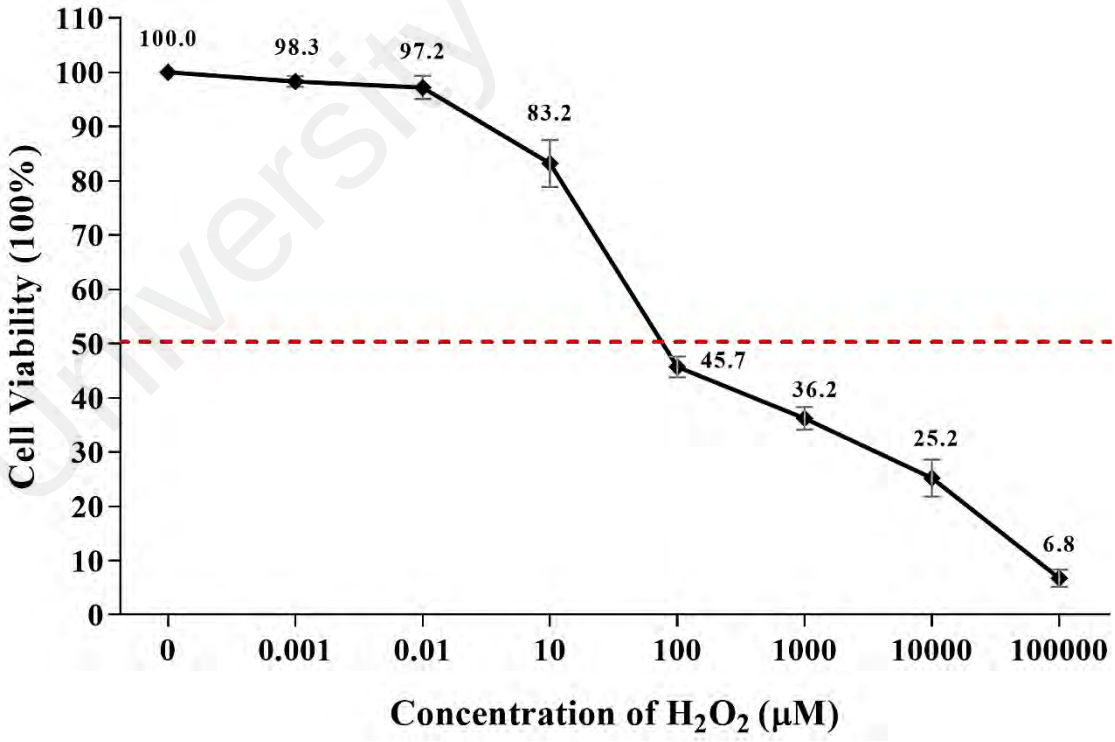


Figure 0.13: Determination of hydrogen peroxide (H_2O_2) assessed by NRU assay. The concentration of H_2O_2 ranges from 0.001–100000 µM was used to induce injury of the NHDF cells. Red line indicates 50% viability of cells.

4.4.2 Determination of Protective Effects

4.4.2.1 Pre-treatment

In the present study, injury to NHDF cells was induced using 100 µM of H_2O_2. As illustrated in Figure 4.14, pre-treatment of NHDF cells with single and combined vegetable extracts resulted in significant protection and a higher percentage of viable cells. At a concentration of 100 µg/ml, the single extract of CA-M exhibited a significant protective effect with a high percentage of cell viability (85.56 ± 3.01%), followed by AI-A (71.35 ± 2.61%), and was comparable with the positive control (ascorbic acid, 100 µg/ml) used in this study (88.99 ± 1.77%). All extract combinations provided cell protection in the range of 55.27%–63.14%.

4.4.2.2 Post-treatment

For post-treatment studies, NHDF cell injury was induced using H_2O_2 for 1 hour before the addition of extracts. As shown in Figure 4.15, post-treatment of NHDF with single and combined vegetable extracts was not able to sustain the survival of the cells. The survival percentage of NHDF cells for all vegetable extracts was not significant, between 46.54% and 52.13%.

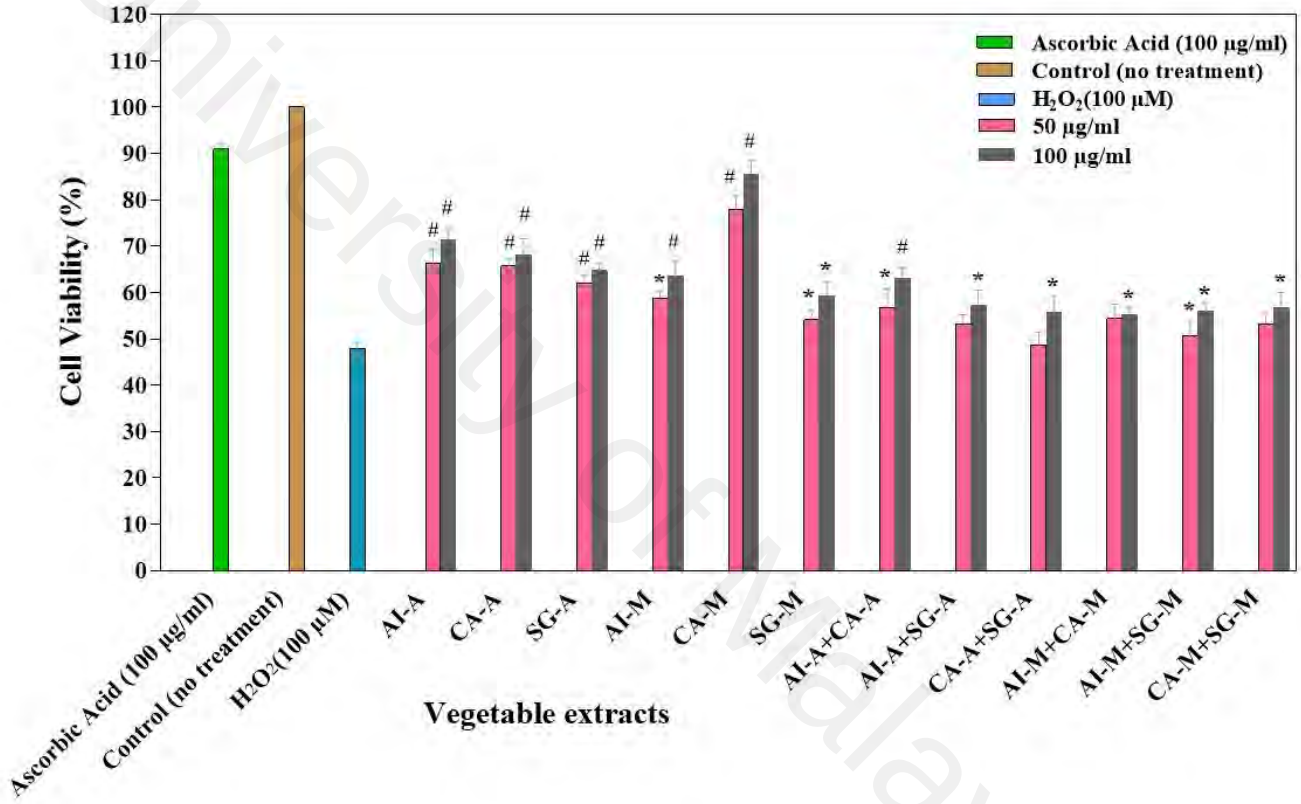


Figure 0.14: Protective effects of single and combined vegetable extracts at 50 and 100 µg/ml. The NHDF cells were treated with the vegetable extracts for 24 hours (pre-treatment), incubated in 5% CO_2 at 37°C. Then, the cells were induced with hydrogen peroxide (H_2O_2) at concentration 100 µM for 1 hour and were assessed via NRU assay at 540 nm wavelength. The results for each point represent the mean ± SD, n=3. # $P < 0.001$ and *$P < 0.05$ versus negative control (H_2O_2 at 100 µM).

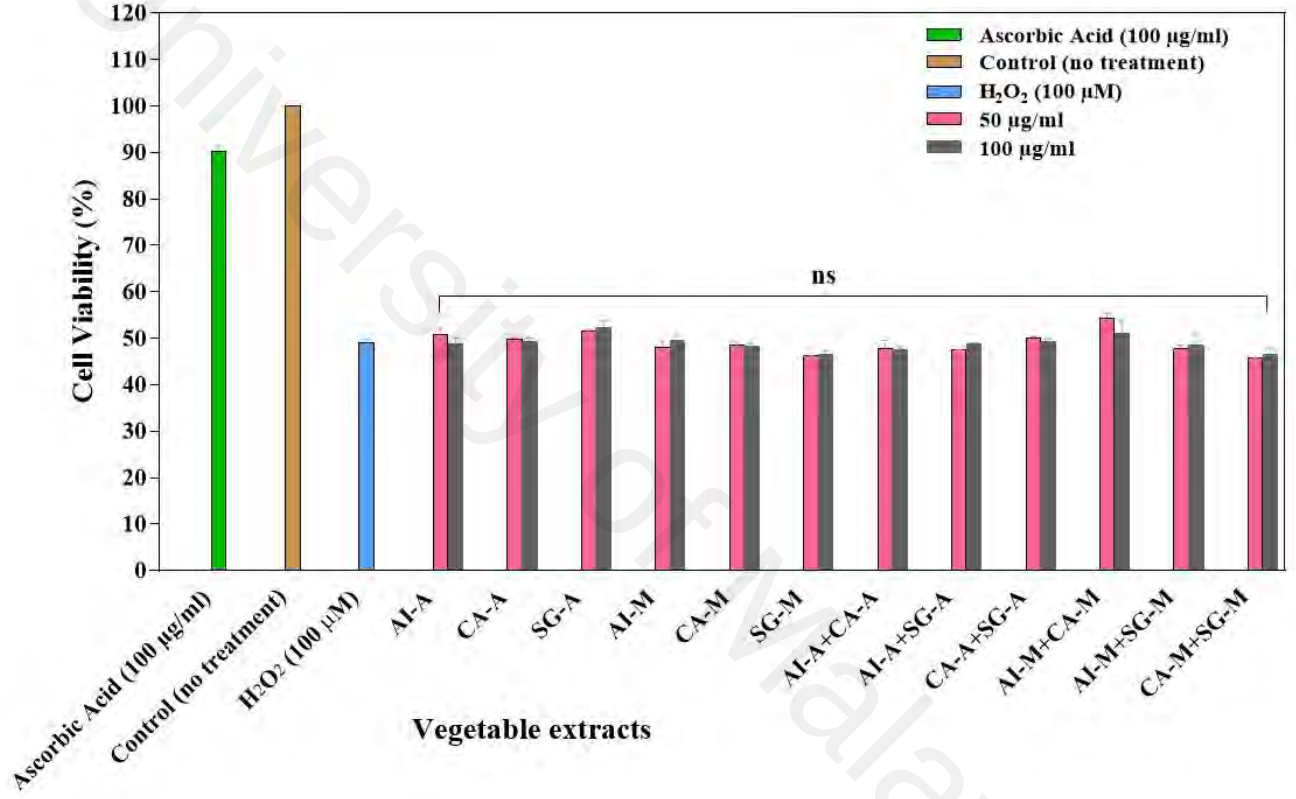


Figure 0.15: Protective effects of single and combined vegetable extracts at 50 and 100 µg/ml. The NHDF cells were induced with hydrogen peroxide (H_2O_2) at concentration 100 µM for 1 hour. Then the cells were treated with the vegetable extracts for 24 hours (post-treatment), incubated in 5% CO_2 at 37°C, and were assessed via NRU assay at 540 nm wavelength. The results for each point represent the mean ± SD, n=3. ns = not significant, # $P < 0.001$ and *$P < 0.05$ versus negative control (H_2O_2 at 100 µM).

4.5 Nitric Oxide (NO) Stimulation Assay

The single and combined vegetable extracts were able to induce nitric oxide (NO) production from murine macrophages cells (RAW264.7) in a dose-dependent manner, in comparison with untreated controls (Figure 4.16). In general, aqueous extracts were better NO stimulator compared to the methanol extracts. Only AI-A and CA-A were able to stimulate NO production significantly. At 25 µg/ml, AI-A and CA-A yielded 2.03 µM and 9.04 µM of NO, respectively. At 100 µg/ml, AI-A and CA-A produced 4.57 µM and 12.36 µM of NO, respectively. At 500 µg/ml, AI-A and CA-A stimulated 5.52 µM and 17.85 µM of NO production, respectively. AI-M stimulated production of 3.11 µM NO at 100 mg/ml and 7.66 µM NO at 500 mg/ml. While no significant effects were observed when RAW264.7 cells treated with SG-A, CA-M, and SG-M (1.10-3.13 µM). NO production stimulator for single vegetable extracts was ranked as follow: CA-A > AI-A > AI-M.

Interestingly, all the combined vegetable extracts were able to stimulate NO production by RAW264.7 cells. At 25 µg/ml, AI-A+CA-A induced 6.75 µM of NO and CA-A+SG-A induced 7.13 µM of NO. At 100 µg/ml, only AI-A+CA-A (13.65 µM) and CA-A+SG-A (13.10 µM) were able to stimulate the production of NO significantly. At 500 µg/ml, AI-A+CA-A (38.89 µM) and CA-A+SG-A (40.84 µM) stimulated the highest production of NO followed by AI-A+CA-M (15.03 µM), AI-M+SG-M (7.28 µM), AI-M+SG-M (6.07 µM), and CA-M+SG-M (5.96 µM). RAW264.7 cells treated with the combination of AI-A+CA-A and CA-A+SG-A produced more NO compared to the positive control, LPS (1 µg/ml = 30.28 µM). NO production stimulator for combined vegetable extracts was ranked as follow: CA-A+SG-A > AI-A+CA-A > AI-A+SG-A > AI-M+CA-M > AI-M+SG-M > CA-M+SG-M.

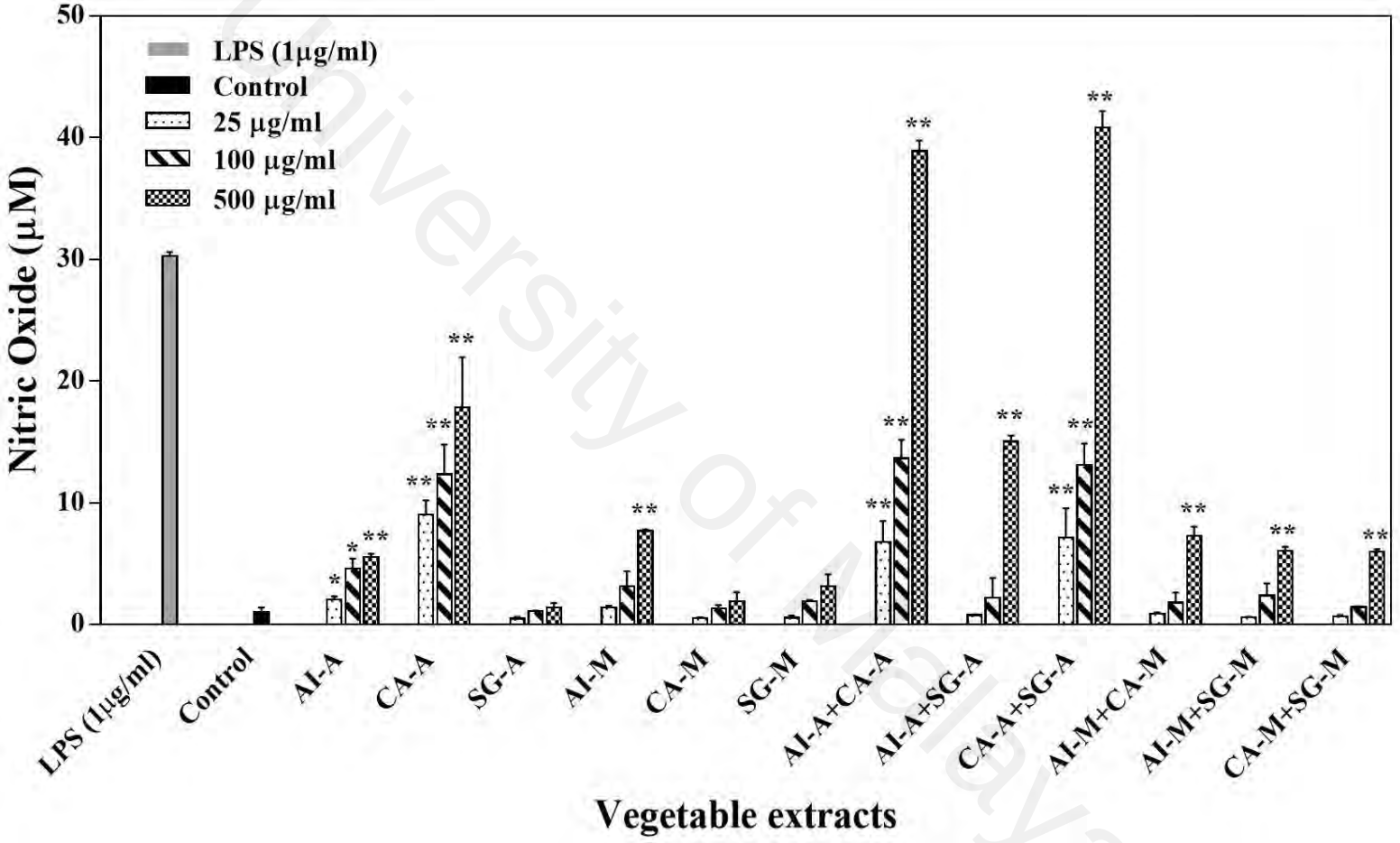


Figure 0.16: Production of nitric oxide (NO) by RAW 264.7 macrophage cell line treated with single and combined vegetable extracts. *$P < 0.001$, **$P < 0.0001$ versus positive control (LPS = 1 µg/ml).

4.5.1 Synergistic Interaction of Combined Vegetable Extracts

Analysis of synergistic interaction of the combined vegetable extracts is shown in Figure 4.17. As the concentration of extract increased (25, 50, and 100 µg/ml), AI-A+CA-A (Figure 4.17 A), CA-A+SG-A (Figure 4.17 C), CA-M+SG-M (Figure 4.17 F) demonstrated synergism (CI < 1) at all combination data points. AI-A+SG-A (Figure 4.17 B) and AI-M+CA-M (Figure 4.17 D) showed antagonism (CI > 1) at 25–50 µg/ml and synergism (CI < 1) at 100 µg/ml). AI-M+SG-M displayed antagonistic (CI < 1) interaction at 25 µg/ml and synergism (CI < 1) at 50–100 µg/ml.

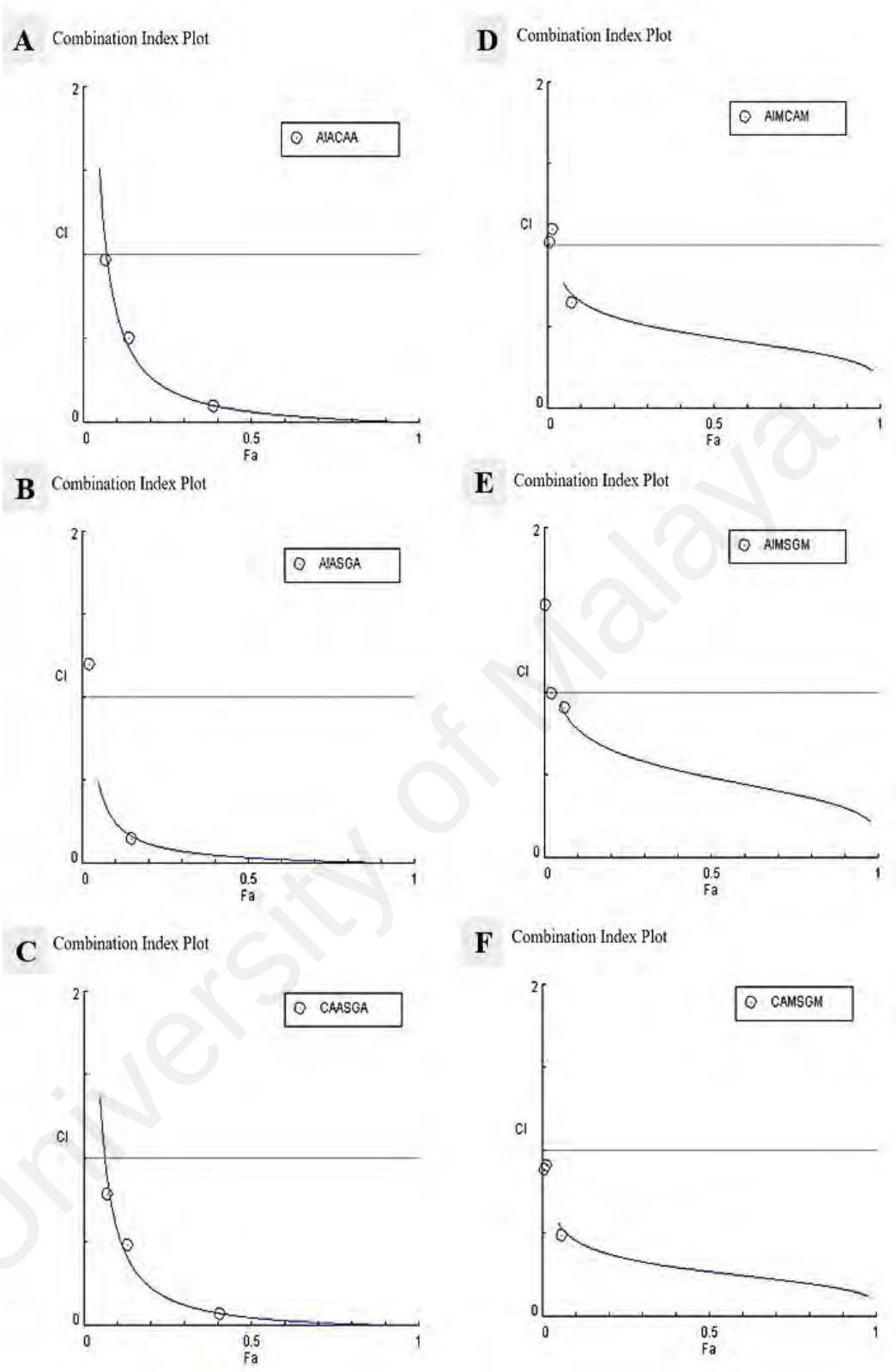


Figure 0.17: Synergism analysis of the combined vegetable extracts (1:1) on stimulation of nitric oxide (NO) production. (A) Combination of AI-A+CA-A; (B) Combination of AI-A+SG-A; (C) Combination of CA-A+SG-A; (D) Combination of AI-M+CA-M; (E) Combination of AI-M+SG-M; and (F) CA-M+SG-M. *CI values were obtained via CompuSyn software where synergism at CI < 1, additive at CI = 0, and antagonism at CI > 1.

4.6 Total Phenolic Content and Antioxidant Properties

The phenolic content, reducing potential, and scavenging properties of the vegetable extracts were evaluated. As indicated in Table 4.9, the most significant and highest total phenolic content (TPC) value was detected in the single extract of AI-A (82.94 ± 1.95 mg GAE/g) and the combined extract of CA-M+SG-M (92.68 ± 7.27 mg GAE/g).

Ferric reducing antioxidant power (FRAP), 2,2-diphenyl-1-picrylhydrazyl (DPPH), and 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assays were performed to investigate the antioxidant activities of single and combined vegetable extracts. As shown in Table 4.9, AI-A had the highest TPC value and thus exhibited the highest reducing potential (61.63 ± 1.48 mM Fe(II)/mg), DPPH free radical scavenging (IC_{50} = 343.46 ± 44.69 µg/ml), and ABTS free radical scavenging (IC_{50} = 473.70 ± 17.01 µg/ml).

Although the combined CA-M+SG-M extract was rich in phenolic content, it did not show the highest antioxidant activities. In fact, it demonstrated low reducing activity (15.49 ± 1.02 mM Fe(II)/mg), low DPPH free radical scavenging activity (3347.25 ± 119.36 µg/ml), and moderate ABTS free radical scavenging activity (706.40 ± 28.12 µg/ml). While the combination of AI-A+SG-A showed the highest ABTS free radical scavenging activity (IC_{50} = 544.53 ± 10.81 µg/ml), the combination AI-A+CA-A had the highest reducing (49.55 ± 0.93 mM Fe(II)/mg) and DPPH free radical scavenging (IC_{50} = 379.75 ± 16.23 µg/ml) properties. However, the antioxidant potential of the extracts was relatively lower compared with that of the positive control (ascorbic acid).

Table 0.9: Antioxidative properties of single and combined vegetable extracts.

Extracts	(mg GAE/g)	(mM Fe (II)/mg)	IC_{50} (µg/ml)	IC_{50} (µg/ml)
	TPC	FRAP	DPPH	ABTS
AI-A	**82.94 ± 1.95^{ef}**	**61.63 ± 1.48^{g}**	**343.46 ± 44.69^{a}**	**473.70 ± 17.07^{a}**
CA-A	79.43 ± 2.58^{de}	39.70 ± 1.55^{e}	661.48 ± 40.98^{a}	606.73 ± 36.93^{bcd}
SG-A	61.21 ± 6.18^{b}	9.03 ± 0.35^{a}	3891.81 ± 405.34^{f}	547.13 ± 46.01^{abcd}
AI-M	44.79 ± 4.21^{a}	13.86 ± 2.28^{ab}	1840.02 ± 53.62^{c}	520.93 ± 15.22^{ab}
CA-M	72.95 ± 2.21^{cde}	17.08 ± 3.16^{b}	2557.67 ± 108.98^{d}	649.33 ± 84.85^{de}
SG-M	69.55 ± 3.62^{bcd}	14.74 ± 2.90^{ab}	7830.99 ± 120.61^{g}	519.27 ± 36.35^{ab}
AI-A+CA-A	74.25 ± 2.51^{cde}	**49.55 ± 0.93^{f}**	**379.75 ± 16.23^{a}**	578.70 ± 6.39^{bcd}
AI-A+SG-A	79.75 ± 3.89^{de}	33.76 ± 0.11^{d}	663.90 ± 41.83^{a}	**544.53 ± 10.81^{abc}**
CA-A+SG-A	80.40 ± 2.46^{de}	23.38 ± 0.12^{c}	1329.81 ± 76.14^{b}	642.87 ± 36.18^{cd}
AI-M+CA-M	66.90 ± 4.98^{bc}	15.29 ± 0.78^{ab}	2042.94 ± 5.37^{c}	571.10 ± 34.74^{abcd}
AI-M+SG-M	73.39 ± 6.05^{cde}	13.73 ± 0.33^{ab}	4299.38 ± 128.69^{f}	555.87 ± 22.76^{abcd}
CA-M+SG-M	**92.68 ± 7.27^{f}**	15.49 ± 1.02^{ab}	3347.25 ± 119.36^{e}	706.40 ± 28.12^{e}
Ascorbic acid	-	765.01 ± 19.32	28.91 ± 0.73	24.08 ± 0.80

AI-A = *A. indica* aqueous extract AI-M = *A. indica* methanol extract
CA-A= *C. asiatica* aqueous extract CA-M = *C. asiatica* methanol extract
SG-A= *S. grandiflora* aqueous extract SG-M = *S. grandiflora* methanol extract
$^{a-g}$ mean values in a column indicate significant differences at $P < 0.05$.

4.6.1 Synergistic Interaction of Combined Vegetable Extracts

4.6.1.1 FRAP Reducing Properties

For antioxidant-reducing properties, different interactions were displayed at each combination data points. AI-A+CA-A (Figure 4.18 A) and CA-M+SG-M (Figure 4.18 F) showed synergy at 0.5 mg/ml (CI < 1) and antagonism at 1.0 – 1.5 mg/ml (CI > 1). CA-A+SG-A (Figure 4.18 C) showed additive interaction at 0.5–1.0 mg/ml (CI = 1) and synergism at 1.5 mg/ml (CI < 1). AI-A+SG-A (Figure 4.18 B) showed synergism at 0.5 mg/ml (CI < 1), additive at 1.0 mg/ml (CI = 0), and antagonistic at 1.5 mg/ml (CI > 1). AI-M+CA-M (Figure 4.18 D) showed synergism at 0.5 and 1.5 mg/ml (CI < 1) and additive at 1.0 mg/ml (CI = 0). AI-M+SG-M (Figure 4.18 E) demonstrated synergism at 0.5 and 1.5 mg/ml (CI < 1) and antagonistic at 1.0 mg/ml (CI > 1).

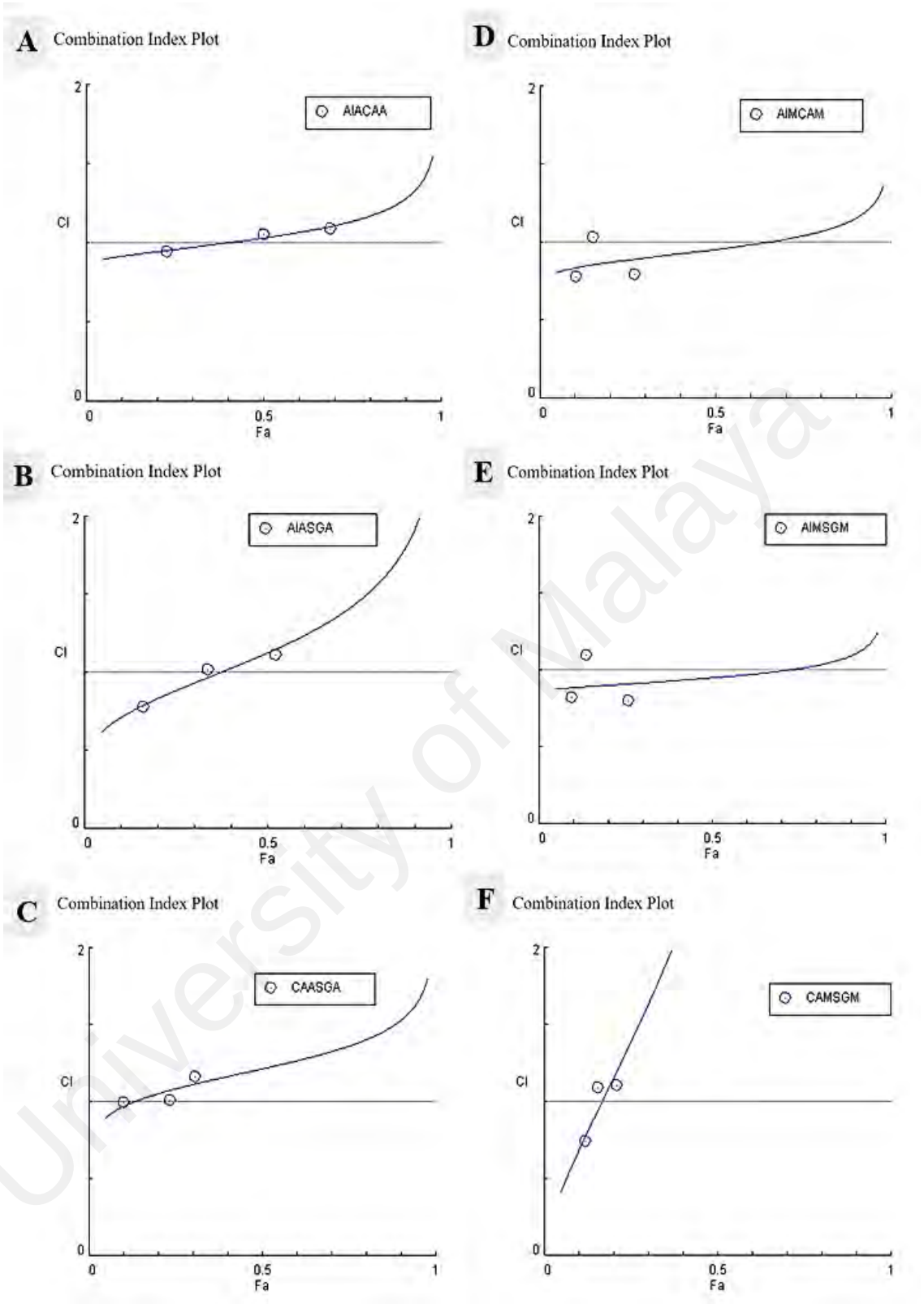


Figure 0.18: Synergism analysis of the combined vegetable extracts (1:1) on ferric reducing activity. (A) Combination of AI-A+CA-A; (B) Combination of AI-A+SG-A; (C) Combination of CA-A+SG-A; (D) Combination of AI-M+CA-M; (E) Combination of AI-M+SG-M; and (F) CA-M+SG-M. *CI values were obtained via CompuSyn software where synergism at CI < 1, additive at CI = 0, and antagonism at CI > 1.

4.6.1.2 DPPH Free Radical Scavenging Activities

Based on the CI-Fa curves in Figure 4.19, each combination data point has different interactions on DPPH free radical scavenging activities. AI-A+CA-A (Figure 4.19 A) displayed synergism at 0.625 – 2.5 µg/ml (CI < 1) and antagonism at 0.313, 5.0, and 10.0 µg/ml (CI > 1). AI-A+SG-A (Figure 4.19 B) and CA-A+SG-A (Figure 4.19 C) showed synergism at 1.25 – 2.5 µg/ml (CI < 1) while antagonism at 0.313–0.625 µg/ml and 5.0–10.0 µg/ml (CI > 1). AI-M+CA-M (Figure 4.19 D) was synergy at 2.5 µg/ml (CI < 1) and antagonism (CI > 1) at other combination data points. AI-M+SG-M (Figure 4.16 E) showed antagonistic interaction (CI > 1). CA-M+SG-M (Figure 4.19 F) showed synergism at 5.0 µg/ml (CI < 1) and antagonism at other combination data points (CI > 1).

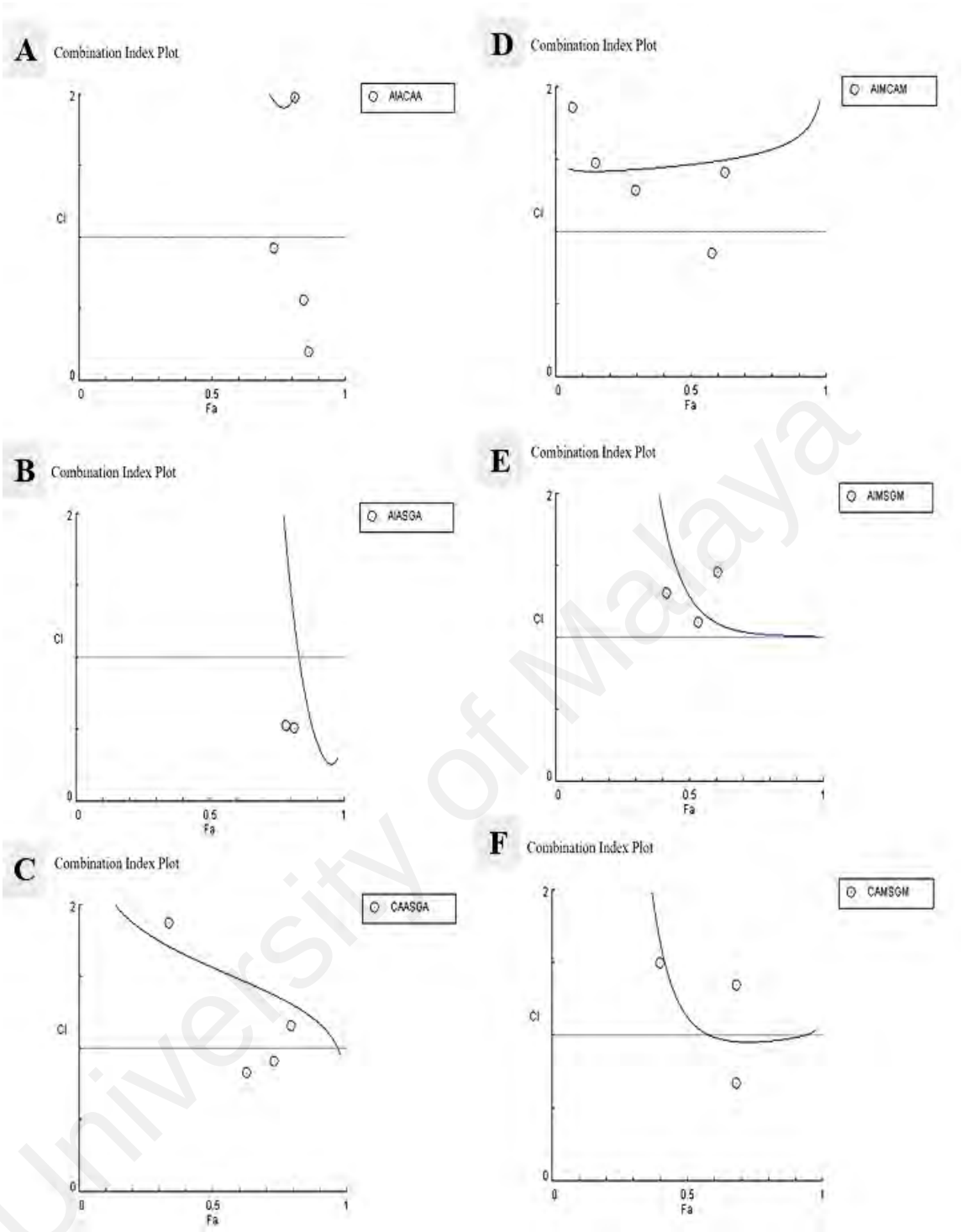


Figure 0.19: Synergism analysis of the combined vegetable extracts (1:1) on DPPH free radical scavenging activities. (A) Combination of AI-A+CA-A; (B) Combination of AI-A+SG-A; (C) Combination of CA-A+SG-A; (D) Combination of AI-M+CA-M; (E) Combination of AI-M+SG-M; and (F) CA-M+SG-M. *CI values were obtained via CompuSyn software where synergism at CI < 1, additive at CI = 0, and antagonism at CI > 1.

4.6.1.3 ABTS Free Radical Scavenging Activities

Various interaction (antagonism, additive, and synergism) of combined vegetable extracts were identified, as shown in Figure 4.20. AI-A+CA-A (Figure 4.20 A) showed synergism at 250, 1250, and 1500 µg/ml (CI < 1) and antagonism at 500–1000 µg/ml (CI > 1). AI-A+SG-A (Figure 4.20 B) demonstrated synergy interaction at 250 and 1500 µg/ml (CI < 1) and antagonism at 500–1250 µg/ml (CI < 1). CA-A+SG-A (Figure 4.20 C) showed antagonism at all combination data points (CI > 1) except additive at 1500 µg/ml (CI = 1). AI-M+CA-M (Figure 4.20 D) showed synergistic interaction as 250 µg/ml (CI < 1) and antagonism at 500–1500 µg/ml (CI > 1). AI-M+SG-M (Figure 4.17 E) showed antagonism at 250–750 µg/ml (CI > 1) and synergism at 1000–1500 µg/ml (CI < 1). CA-M+SG-M (Figure 4.20 F) showed antagonistic interaction (CI > 1) at all combination data points.

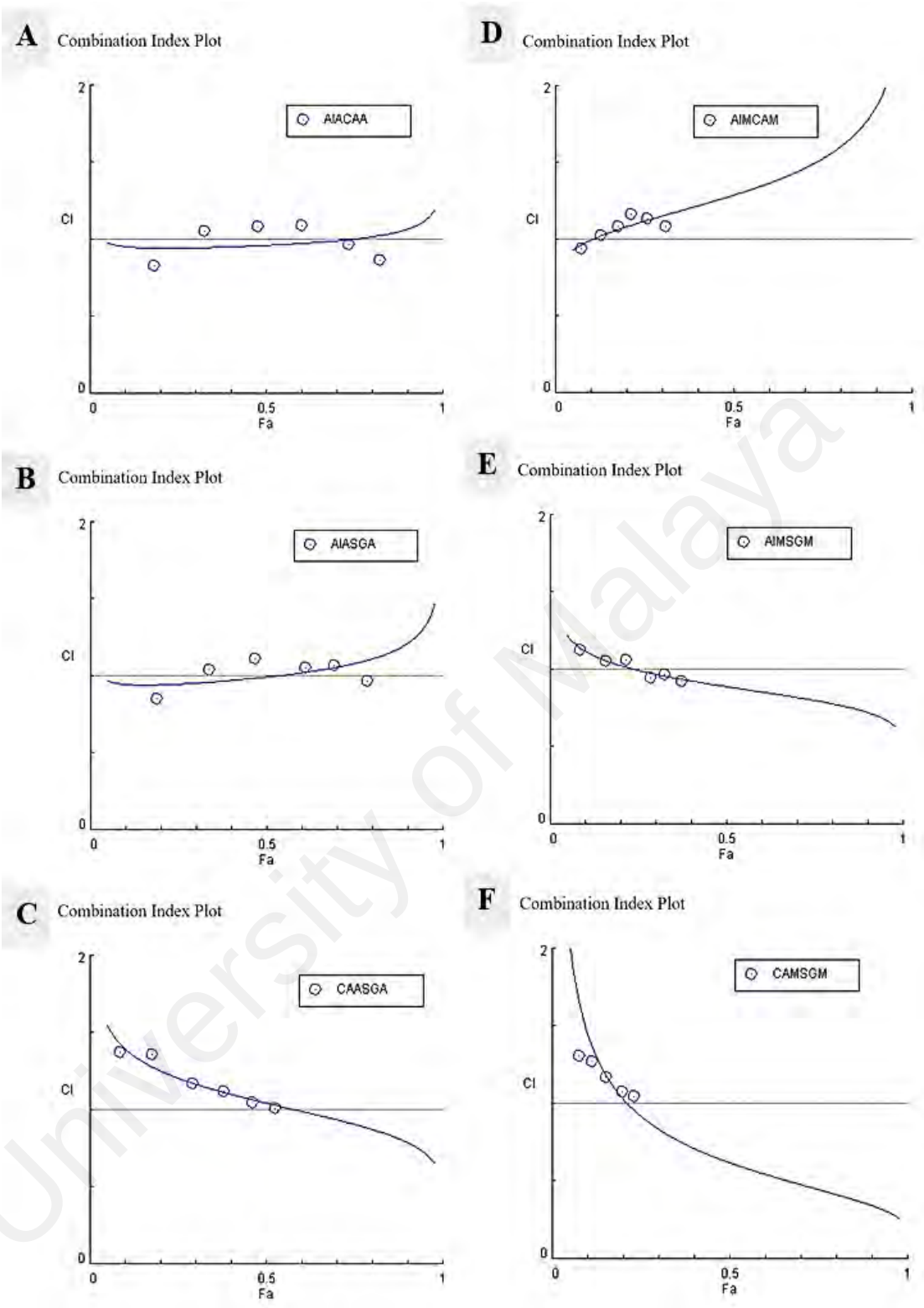


Figure 0.20: Synergism analysis of the combined vegetable extracts (1:1) on ABTS free radical scavenging activities. (A) Combination of AI-A+CA-A; (B) Combination of AI-A+SG-A; (C) Combination of CA-A+SG-A; (D) Combination of AI-M+CA-M; (E) Combination of AI-M+SG-M; and (F) CA-M+SG-M. *CI values were obtained via CompuSyn software where synergism at CI < 1, additive at CI = 0, and antagonism at CI > 1.

4.7 Overall Interaction of Combined Vegetable Extracts

CI values for the combined extracts were obtained by CompuSyn software where synergism at CI < 1, additive at CI = 0, and antagonism at CI > 1. AI-A+CA-A showed synergism for fibroblast migration, DPPH free radical scavenging activity, and production of NO. AI-A+SG-A and CA-A+SG-A demonstrated synergism towards DPPH free radical scavenging activity and production of NO. AI-M+CA-M and AI-M+SG-M showed synergistic interaction for FRAP reducing activity and production of NO. CA-M+SG-M only exhibited synergism for production of NO. Table 4.10 summarized the synergy of the combined vegetable extracts, for each bioactivity, based on the calculated CI value.

Table 0.10: Interaction of the combined vegetable extracts based on the CI value.

Extract	Fibroblast migration	Antibacterial activity	Antifungal activity	Antioxidant properties			Production of NO
				FRAP	DPPH	ABTS	
AI-A + CA-A	Synergism	Antagonism	NA[#]	Antagonism	Synergism[*]	Antagonism[**]	Synergism
AI-A + SG-A	Antagonism	Antagonism	NA	Antagonism	Synergism[*]	Antagonism[**]	Synergism
CA-A + SG-A	Antagonism	Antagonism	NA	Additive	Synergism[*]	Antagonism[**]	Synergism
AI-M + CA-M	Antagonism	Antagonism	Antagonism	Synergism	Antagonism	Antagonism[**]	Synergism
AI-M + SG-M	Antagonism	Antagonism	Antagonism	Synergism	Antagonism	Antagonism[**]	Synergism
CA-M + SG-M	Antagonism	Antagonism	Antagonism	Antagonism	Antagonism	Antagonism[**]	Synergism

AI-A = *Acalypha indica* aqueous extract
CA-A = *Centella asiatica* aqueous extract
SG-A = *Sesbania grandiflora* aqueous extract
AI-M = *Acalypha indica* methanol extract
CA-M = *Centella asiatica* methanol extract
SG-M = *Sesbania grandiflora* methanol extract
Synergism (CI < 1), additive (CI = 0), and antagonism (CI > 1).
#NA = Not Available
*Synergistic interaction at a lower dosage (0.625–2.5 µg/ml) and antagonistic at higher dosage (> 10.0 µg/ml)
**Antagonistic interaction at tested dosage (250–1500 µg/ml), but as the dosage increased the CI values less than 1 (CI < 1) indicate synergism at a higher dosage

4.8 Correlation Between Migration of Fibroblast, Antioxidant, Protectivity, and NO Stimulation

Pearson's correlation analyses (Table 4.11) were done to study the relationship between the rate of fibroblast migration treated with single and combined vegetable extracts with antioxidant properties that were based on TPC (phenolic content), FRAP (reducing potential), DPPH, and ABTS assay (free radical scavenging potential), protective activities, and production of NO. The coefficient correlation (r) that close to 1 or -1 has a strong correlation. There was a significant positive correlation between the rate of fibroblast with reducing potential (r = 0.8264). Migration of fibroblast showed a moderate positive correlation, but not significant, with protectivity against H_2O_2 (r = 0.4607) and production of NO (r = 0.4236). Whereas, an inverse relationship (negative correlation) between migration of fibroblast with DPPH (r = -0.5622) and ABTS (r = -0.2272) free radical scavenging properties. TPC showed no correlationship (r = 0.0852), thus might be not the main contributor for migration of fibroblast.

Table 0.11: Pearson's correlation coefficients (r) between wound healing and other bioactivities.

		Antioxidant potential				Protectivity against H_2O_2	NO production
		TPC	FRAP	DPPH	ABTS		
Fibroblast migration)	Pearson's correlation (r)	0.0852	0.8264*	-0.5622	-0.2272	0.4607	0.4236
	Sig. (2-tailed)	0.792	< 0.001	0.057	0.478	0.132	0.170
	n	12	12	12	12	12	12

*significant at $P < 0.05$
n = number of pairs

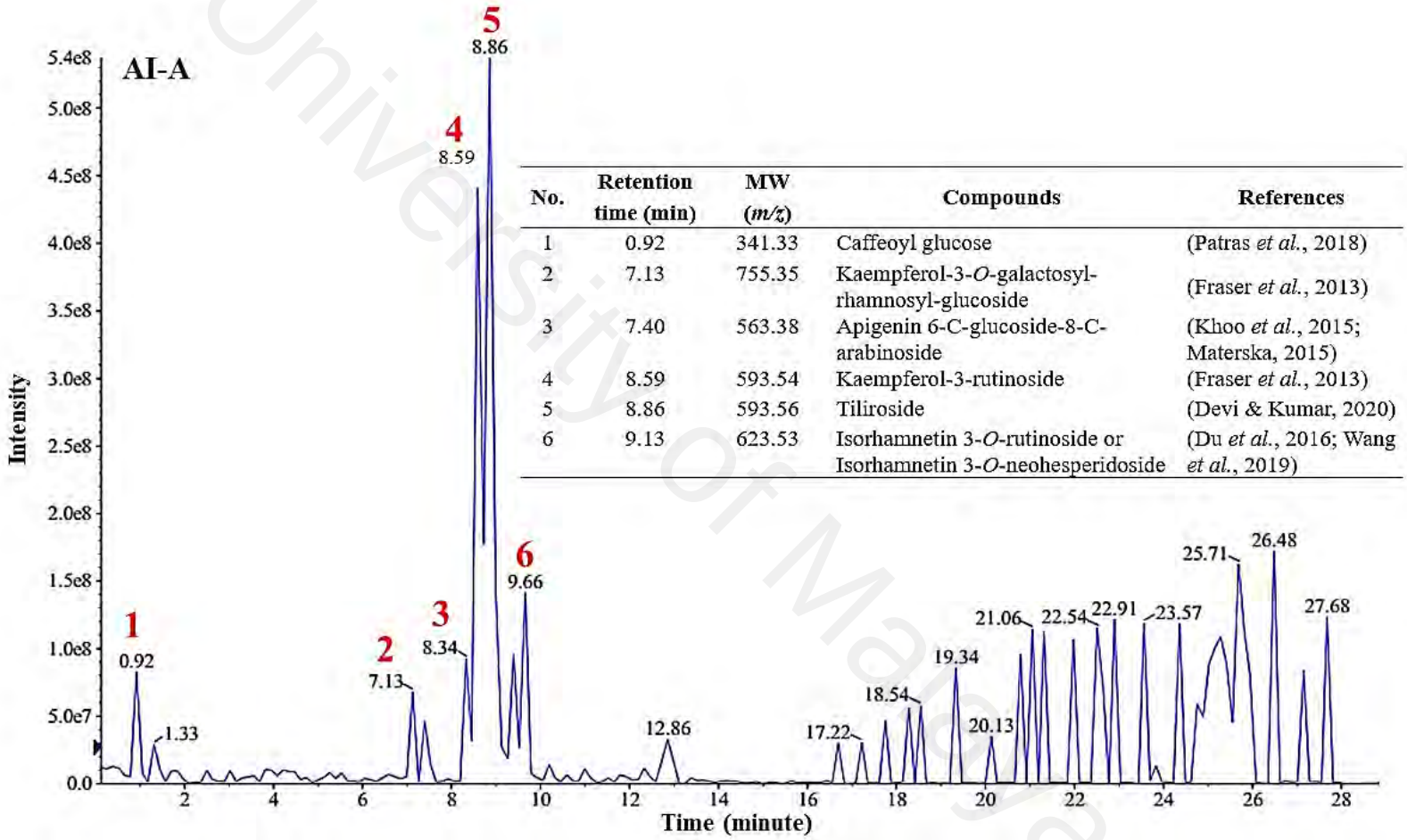
4.9 Chemical Profiling of Vegetable Extracts

The investigation of the compound was performed using liquid chromatography system, UHPLC-QTrap-MS/MS and UHPLC-TWIMS-QTOF-MS/MS. Identification of compound based on their molecular ion mass, retention time, and fragmentation pattern. Figure 4.21 shows the chromatogram for AI-A and six compounds were identified, caffeoyl glucose **(1)**, kaempferol-3-*O*-galactosyl-rhamnosyl-glucoside **(2)**, apigenin 6-C-glucoside-8-C-arabinoside **(3)**, kaempferol-3-rutinoside **(4)**, tiliroside **(5)**, and isorhamnetin 3-*O*-rutinoside or isorhamnetin 3-*O*-neohesperidoside **(6)**. Figure 4.22 shows the full chromatographic separation of AI-M, with three compounds identified: methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate **(1)**, 3,30-di-*O*-methyl ellagic acid **(2)**, and 2(3,4-dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid **(3)**.

Figure 4.23 shows the chromatogram form CA-A and six compounds were identified, quinic acid **(1)**, caffeoylquinic acid derivatives **(2, 3,** and **7),** quercetin-3'-glucuronide **(4),** kaempferol **(5)**, and luteolin-7-*O*-glucoronide isomer **(6)**. Figure 4.24 and Figure 4.25 shows the full chromatogram for CA-M at low collision energy and high collision energy, respectively and 23 compounds were identified, mannotriose **(1)**, isomaltose, pentose, methylsuccinic acid, mono-ethyl fumarate, galactose, sodium ferulate, α-kojibiose **(2)**, genkwadaphnin **(3)**, paeonilactone C1 **(4)**, 5′-methoxy-bilobetin, medicagol **(5)**, roseoside 1 **(6)**, 7-*O*-[4′-*O*-(3″,4″-Dihydroxycinnamyl)-β-D-glucopyranosyl]-6-methoxycoumarin **(7)**, cimicifugic acid B **(8)**, 7-hydroxy-1-methoxy-2-methoxyxanthone, kaempferol 3-*O*-β-D-glucuronopyranoside **(9)**, 7-*O*-[4′-*O*-(3″,4″-Dihydroxycinnamyl)-β-D-glucopyranosyl]-6-methoxycoumarin **(10)**, erigoster A **(11)**, myrianthic acid, nevadensin-5-*O*-β-D-glucoside, madecassoside, protoneogracillin, anemarsaponin F, hypoglaucin G **(12)**, clinopodiside A **(13)**, asiaticoside **(14)**, purpurin,

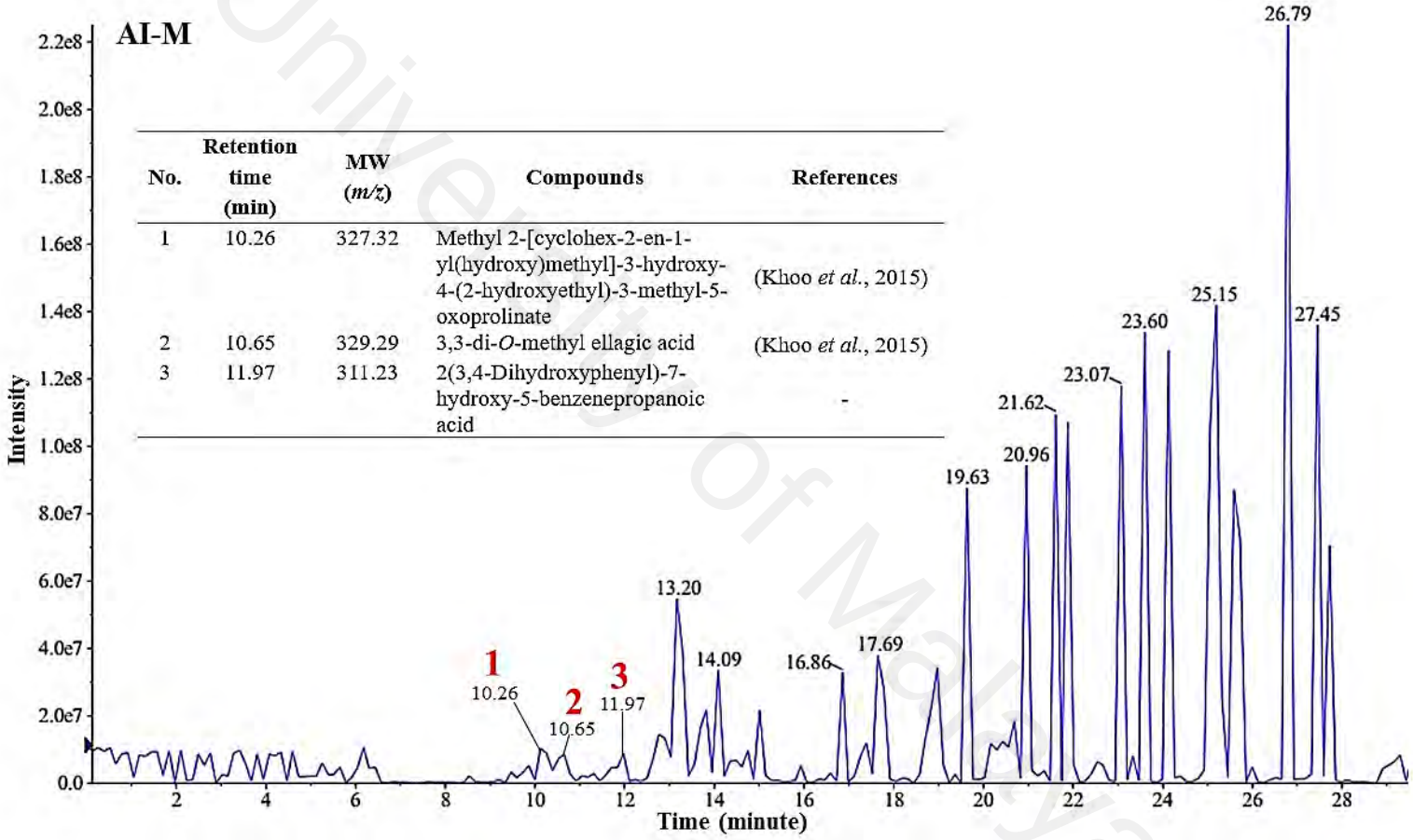
psammosilenins B, esculentoside I, julibroside B1 **(15)**, luteolinidin **(16)**, pedunculagin 1, 16-oxo alisol A **(17)**, quercetin-3-sulphate, 11α,12α-Epoxy-23β,23-dihydroxyolean-28,13β-olide **(18)**, alisol F, 6-gingerol **(19)**, carthamin **(20)**, ciryneone F **(21)**, picrasidine K, magnocurarine, (-)-Chebulic acid triethyl ester **(22)**, and daturametelin J **(23)**.

The 12 compounds identified for SG-A are shown in Figure 4.26, and were as follows: caffeoyl glucose **(1)**, ferulic acid **(2)**, ferulic acid derivatives **(3** and **4)**, kaempferol-rhamnose-hexose-rhamnose derivatives **(5** and **7)**, quercetin-rhamnose-hexose-rhamnose **(6)**, kaempferol-3-*O*-(2”- or 3”-acetyl) rhamnoside derivatives **(8** and **9)**, kaempferol-3-rutinoside **(10)**, *p*-coumaric acid **(11)**, and ferulic acid isomer **(12)**. For SG-M, two compounds were identified (Figure 4.27): methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate **(1)** and 2(3,4-dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid **(2)**.



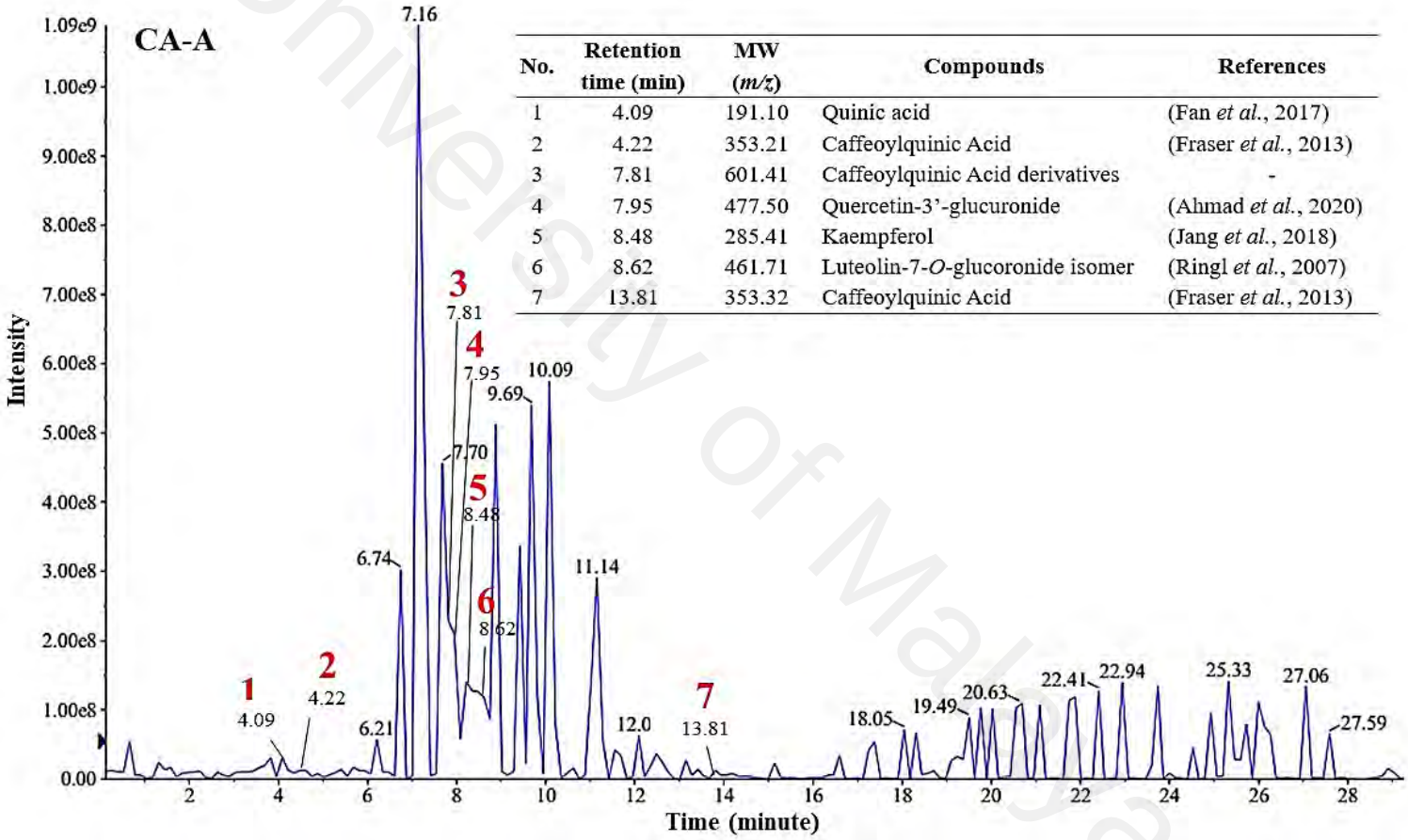
No.	Retention time (min)	MW (*m/z*)	Compounds	References
1	0.92	341.33	Caffeoyl glucose	(Patras *et al.*, 2018)
2	7.13	755.35	Kaempferol-3-*O*-galactosyl-rhamnosyl-glucoside	(Fraser *et al.*, 2013)
3	7.40	563.38	Apigenin 6-C-glucoside-8-C-arabinoside	(Khoo *et al.*, 2015; Materska, 2015)
4	8.59	593.54	Kaempferol-3-rutinoside	(Fraser *et al.*, 2013)
5	8.86	593.56	Tiliroside	(Devi & Kumar, 2020)
6	9.13	623.53	Isorhamnetin 3-*O*-rutinoside or Isorhamnetin 3-*O*-neohesperidoside	(Du *et al.*, 2016; Wang *et al.*, 2019)

Figure 0.21: Chromatogram of the aqueous extract of *A. indica* (AI-A) by using UHPLC-QTrap-MS/MS system in the negative ionic mode.



No.	Retention time (min)	MW (*m/z*)	Compounds	References
1	10.26	327.32	Methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate	(Khoo *et al.*, 2015)
2	10.65	329.29	3,3-di-*O*-methyl ellagic acid	(Khoo *et al.*, 2015)
3	11.97	311.23	2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid	-

Figure 0.22: Chromatogram of the methanol extract of *A. indica* (AI-M) by using UHPLC-QTrap-MS/MS system in the negative ionic mode.



No.	Retention time (min)	MW (*m/z*)	Compounds	References
1	4.09	191.10	Quinic acid	(Fan *et al.*, 2017)
2	4.22	353.21	Caffeoylquinic Acid	(Fraser *et al.*, 2013)
3	7.81	601.41	Caffeoylquinic Acid derivatives	-
4	7.95	477.50	Quercetin-3'-glucuronide	(Ahmad *et al.*, 2020)
5	8.48	285.41	Kaempferol	(Jang *et al.*, 2018)
6	8.62	461.71	Luteolin-7-*O*-glucoronide isomer	(Ringl *et al.*, 2007)
7	13.81	353.32	Caffeoylquinic Acid	(Fraser *et al.*, 2013)

Figure 0.23: Chromatogram of the aqueous extract of *C. asiatica* (CA-A) by using UHPLC-QTrap-MS/MS system in the negative ionic mode.

No.	Retention time (min)	MW (*m/z*)	Compounds	References
3	0.59	601.21	Genkwadaphnin	(Tao *et al.*, 2018)
5	0.96	581.11	5′-Methoxy-bilobetin	-
	0.96	295.03	Medicagol	(Zhang *et al.*, 2014)
6	6.46	385.19	Roseoside_1	(Turghun *et al.*, 2018)
8	9.29	447.09	Cimicifugic acid B	(Yim *et al.*, 2012)
10	9.66	515.12	7-*O*-[4′-*O*-(3″,4″-Dihydroxycinnamyl)-β-D-glucopyranosyl]-6-methoxycoumarin	-
14	13.53	957.51	Asiaticoside	(Gupta *et al.*, 2018)
15	13.99	255.03	Purpurin	(Lee *et al.*, 2015)
	13.99	857.45	Psammosilenins B	(Hou *et al.*, 2020)
	13.99	1017.49	Esculentoside I	-
	13.99	971.48	Julibroside B1	-
16	16.55	305.02	Luteolinidin	(Wu *et al.*, 2016)
18	16.63	380.99	Quercetin-3-sulphate	(Wang *et al.*, 2015)
	16.63	501.32	11α,12α-Epoxy-23β,23-dihydroxyolean-28,13β-olide	-
20	16.66	909.21	Carthamin	(Guo *et al.*, 2017)
22	16.70	312.17	Picrasidine K	-
	16.70	313.17	Magnocurarine	(Li *et al.*, 2017)
	16.70	439.13	(-)-Chebulic acid triethyl ester	(Yang *et al.*, 2020)

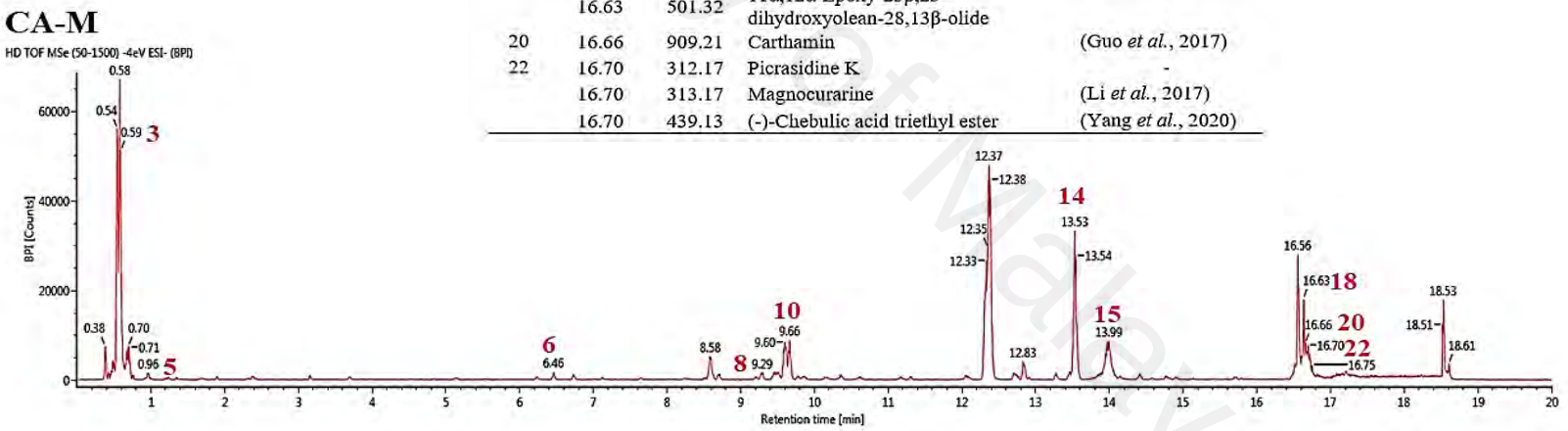


Figure 0.24: Chromatogram of the methanol extract of *C. asiatica* (CA-M) by using UHPLC-TWIMS-QTOF-MS/MS system at low collision energy (CE of 4 eV).

No.	Retention time (min)	MW (*m/z*)	Compounds	References
1	0.54	503.16	Mannotriose	(Jonathan *et al.*,
2	0.58	341.11	Isomaltose	2017; Scorrano *et*
	0.58	149.05	Pentose	*al.*, 2017; Kiryu *et*
	0.58	131.03	Methylsuccinic acid	*al.*, 2020)
	0.58	143.03	Mono-ethyl fumarate	-
	0.58	179.06	Galactose	(Beus *et al.*, 2019)
	0.58	215.03	Sodium ferulate	(Zhao *et al.*, 2019)
	0.58	341.11	α-Kojibiose	-
4	0.70	317.10	Paeonilactone C	-
7	9.28	515.12	7-*O*-[4′-*O*-(3″,4″-Dihydroxycinnamyl)-β-D-glucopyranosyl]-6-methoxycoumarin	(Cui *et al.*, 2020)
9	9.60	285.04	7-Hydroxy-1-methoxy-2-methoxyxanthone	-
	9.60	461.07	Kaempferol 3-*O*-β-D-glucuronopyranoside	(Alara *et al.*, 2020)
11	9.67	557.13	Erigoster A	(Tartaglione *et al.*, 2018)
	9.67	353.09	4-*O*-Caffeoylquinic acid	(Huang *et al.*, 2019)
12	12.37	503.34	Myrianthic acid	(Willems *et al.*, 2016)
	12.37	505.14	Nevadensin-5-*O*-β-D-glucoside	(Alara *et al.*, 2020)
	12.37	973.5	Madecassoside	-
	12.37	1063.53	Protoneogracillin	(Rani *et al.*, 2020)
	12.37	1049.52	Anemarsaponin F	(Ling *et al.*, 2020)
	12.37	1061.52	Hypoglaucin G	(Zhong *et al.*, 2017)
13	12.84	957.51	Clinopodiside A	(Zhang *et al.*, 2016)
14	13.53	957.51	Asiaticoside	(Zeng *et al.*, 2016)
16	16.55	305.02	Luteolinidin	(Gupta *et al.*, 2018)
19	16.64	487.34	Alisol F	(Wu *et al.*, 2016)
	16.64	293.18	6-Gingerol	(Liu *et al.*, 2016)
21	16.68	275.17	Ciryneone F	(Zhang *et al.*, 2017)
23	18.52	631.31	Daturametelin J	-

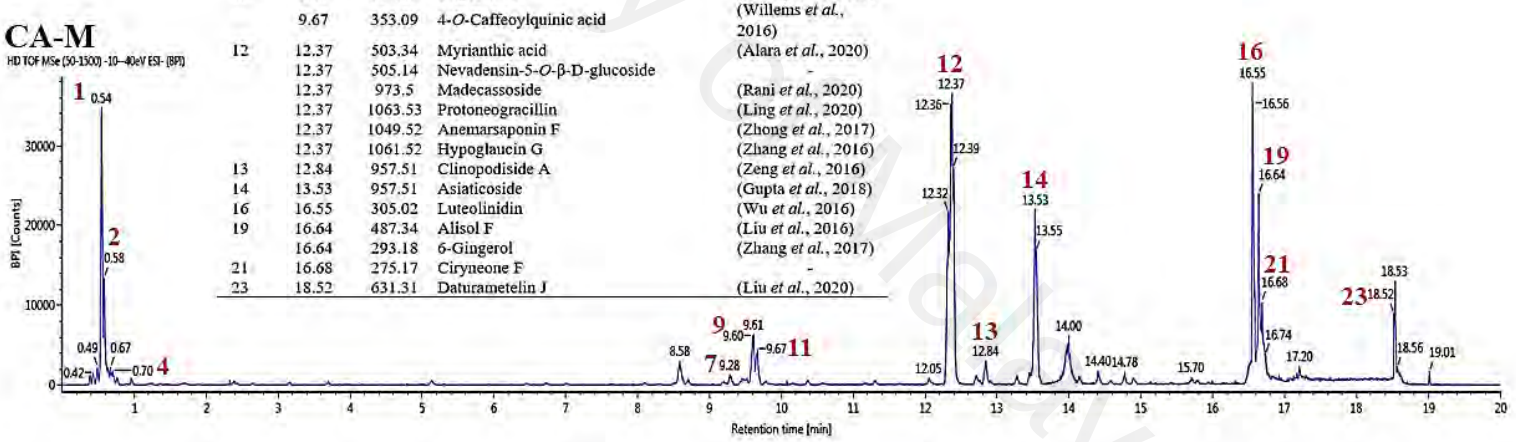
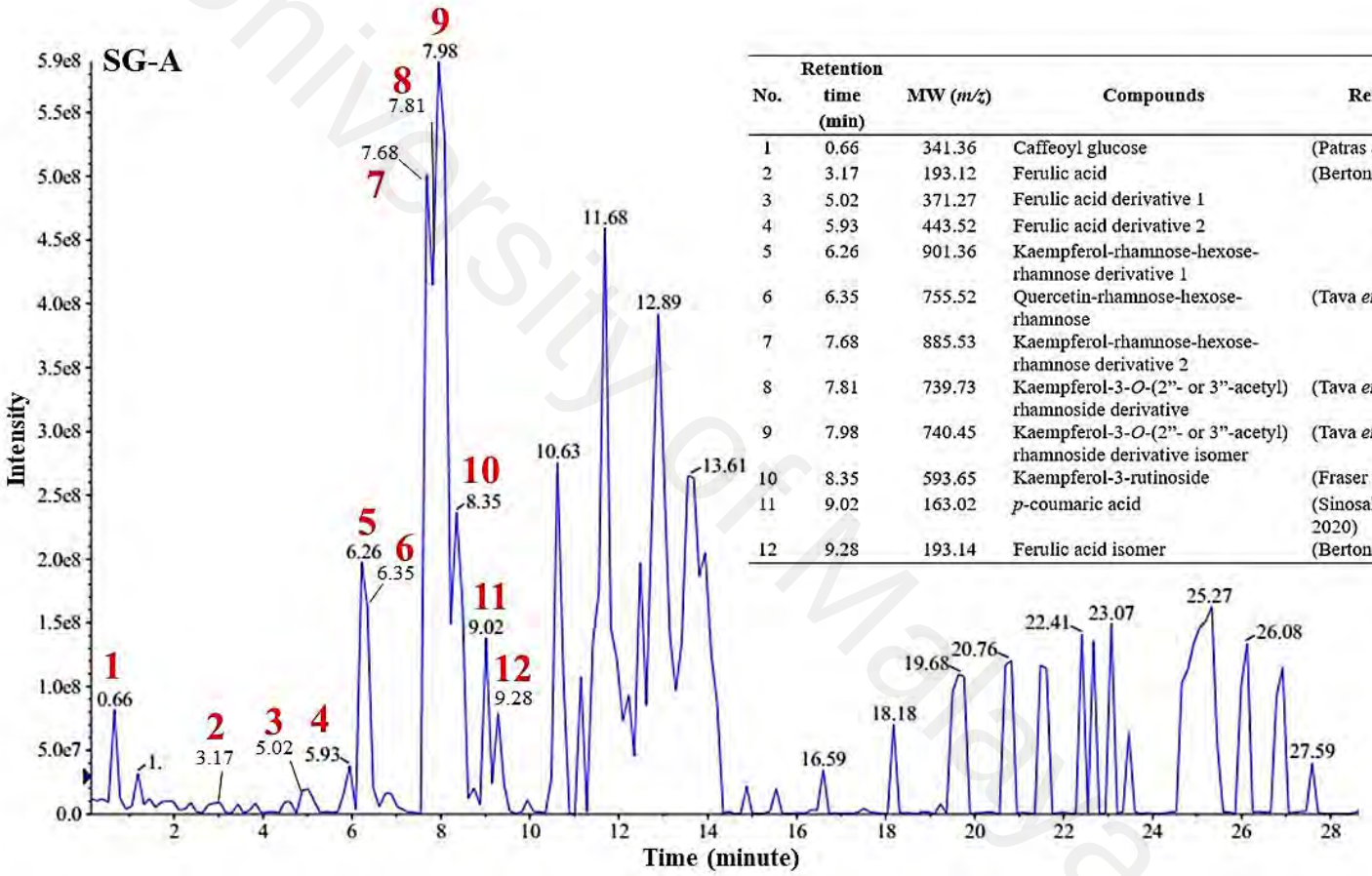
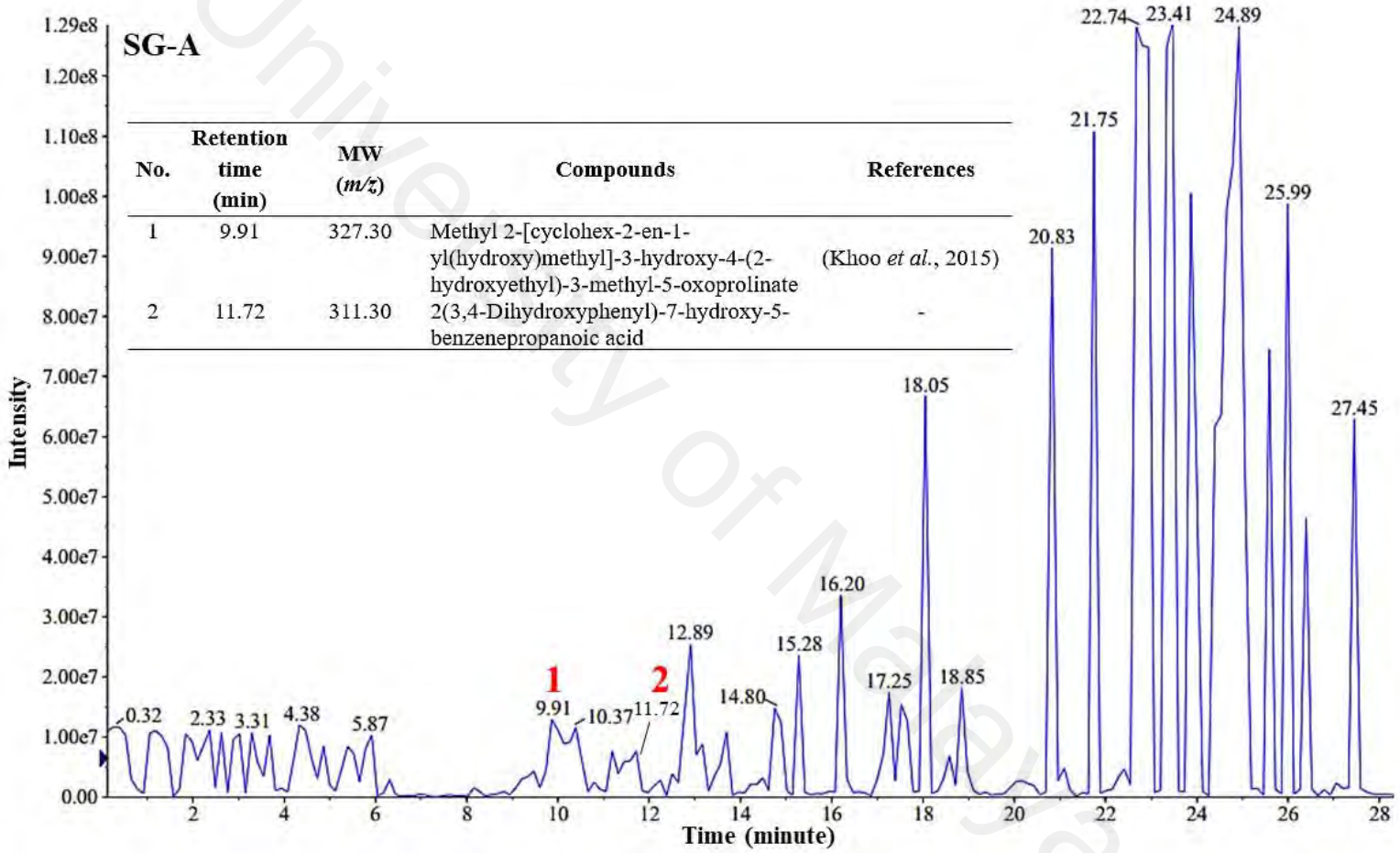


Figure 0.25: Chromatogram of the methanol extract of *C. asiatica* (CA-M) by using UHPLC-TWIMS-QTOF-MS/MS system at high collision energy (CE of 10–40eV).



No.	Retention time (min)	MW (*m/z*)	Compounds	References
1	0.66	341.36	Caffeoyl glucose	(Patras *et al.*, 2018)
2	3.17	193.12	Ferulic acid	(Berton *et al.*, 2020)
3	5.02	371.27	Ferulic acid derivative 1	-
4	5.93	443.52	Ferulic acid derivative 2	-
5	6.26	901.36	Kaempferol-rhamnose-hexose-rhamnose derivative 1	-
6	6.35	755.52	Quercetin-rhamnose-hexose-rhamnose	(Tava *et al.*, 2019)
7	7.68	885.53	Kaempferol-rhamnose-hexose-rhamnose derivative 2	-
8	7.81	739.73	Kaempferol-3-*O*-(2"- or 3"-acetyl) rhamnoside derivative	(Tava *et al.*, 2019)
9	7.98	740.45	Kaempferol-3-*O*-(2"- or 3"-acetyl) rhamnoside derivative isomer	(Tava *et al.*, 2019)
10	8.35	593.65	Kaempferol-3-rutinoside	(Fraser *et al.*, 2013)
11	9.02	163.02	*p*-coumaric acid	(Sinosaki *et al.*, 2020)
12	9.28	193.14	Ferulic acid isomer	(Berton *et al.*, 2020)

Figure 0.26: Chromatogram of the aqueous extract of *S. grandiflora* (SG-A) by using UHPLC-QTrap-MS/MS system in the negative ionic mode.



No.	Retention time (min)	MW (*m/z*)	Compounds	References
1	9.91	327.30	Methyl 2-[cyclohex-2-en-1-yl(hydroxy)methyl]-3-hydroxy-4-(2-hydroxyethyl)-3-methyl-5-oxoprolinate	(Khoo *et al.*, 2015)
2	11.72	311.30	2(3,4-Dihydroxyphenyl)-7-hydroxy-5-benzenepropanoic acid	-

Figure 0.27: Chromatogram of the methanol extract of *S. grandiflora* (SG-M) by using UHPLC-QTrap-MS/MS system in the negative ionic mode.

DISCUSSION

5.1 The Importance of Plant Identification

Establishing the botanical identity and correct taxonomy name to the plant sample is the most fundamental procedure in any plant-related research (Bennett & Balick, 2008). Scientific botanical name (genus and species are written in *Italic*) as 'plant passport' that serves as a unique identifier for a plant that contains vast information regarding the plant (Nesbitt et al., 2010). The development of plant-derived drugs is a multidisciplinary effort that involves experts from different fields; such as ethnobotanist, toxicologist, plant taxonomist, phytochemist, pharmacologist, medicinal chemist, and others; data of the plant should be conveyed across language, scientific fields, and electronic retrieval system without any problem (Bennett & Balick, 2014).

The common taxonomic error made by researchers are incorrect family assignment and author citations of scientific names, misspelt epithet and generic names, and use of synonyms instead of currently accepted names (Chan et al., 2012). Nesbitt et al. (2010) reviewed 50 publication that listed 502 sample plants and 27% of the plant names were not up-to-date and misspelt. Because of the errors, researchers cannot retrieve accurate information from existing literature, thus reduce or invalidate the value of the research findings (Rivera et al., 2014). Production of an unambiguous document of a plant species with correct scientific name must be linked to a voucher or herbarium specimen collected during fieldwork followed by deposition on one or more herbaria. A voucher specimen is essential to secure initial identification (ID) by comparing with other herbarium specimens. If ID is questioned, the original plant can be re-examined to confirm the identity (Nesbitt et al., 2010).

In this study, *Acalypha indica* (AI), *Centella asiatica* (CA), and *Sesbania grandiflora* (SG) were collected from a designated location in Peninsular Malaysia. The plant specimens were dried and preserved, carefully mounted onto herbarium sheet, assigned with the current scientific names and were given voucher numbers. Authentication of the plant specimens was carried out at the herbarium of Rimba Ilmu Botanical Garden, Institute of Biological Sciences and the voucher materials were deposited at the same herbarium.

5.2 Methods of Vegetable Extraction

Fresh and dried leaves of *A. indica*, *C. asiatica*, and *S. grandiflora* were used in this study. A fresh sample is ideal for phytochemical analysis because it retains the originality of its condition without altering its phytochemical contents. As explained in Tiwari et al. (2013) after plant sample is harvested, it must be kept at low temperature, and experimental work must be done quickly since the fresh sample is fragile and can easily deteriorate in their quality. Alternatively, dried plants were used for extraction to preserve desirable qualities (oven-dried at 35ºC) and the samples remain in good condition for a more extended period. Vongsak et al. (2013), demonstrated in their study that oven drying showed no significant effect in total phenolic content but have higher flavonoids content in dried *Moringa oliefera* leaves (drumstick tree) compared to the fresh leaves.

Extraction of phytochemicals depend on the solvent used throughout the procedure, and several factors need to be considered when choosing the solvent or solvent system, i.e., the safety of the solvent, extraction or formation of unwanted compounds, and solubility of the desirable compounds (Thouri et al., 2017). In this study, methanol and water were solvents used for phytochemical extraction. Methanol permits maximum extraction and produces high yield phytochemical extraction yields. However, methanol

is harmful if ingested by human, and it is not suitable for consumption. Hence, the methanol must be obliterated from the extracts before incorporating into foods and beverages. The phytochemical groups that can be extracted by using methanol were phenolic compounds (phenols, phenolic acids, anthocyanins, and flavonoids), terpenoids (essential oils, carotenoids, diterpenoids, and triterpenoids) lipids, alkaloids, (Harborne, 1999). Water is a commonly used solvent in traditional practices for plant phytochemical extraction. It is safe for human consumption, and extracted compounds are almost similar to the organic solvent with additional polysaccharides (Tiwari et al., 2013; Liu et al., 2015a).

5.3 Wound Healing Activity of the Vegetable Extracts

In this experiment, the migration of fibroblast cells were evaluated via *in vitro* scratch assay in which an artificial gap (representing a wound) is created by scratching on a confluent monolayer cell. Wound closure was analyzed by post-wounding static imaging. The images were captured at 0 hour (T0), 24 hour (T24), and 48 hour (T48). The *in vitro* scratch assay is an easy and affordable method. Yet, it is a well-developed method that is appropriate for investigations on the effects of various cell-matrix interactions on cell migration and mimics migration of cells during wound healing in *in vivo* model (Liang et al., 2007; Brusotti et al., 2015).

The current study indicated that single aqueous vegetable extract of CA-A and combined aqueous vegetable extract of AI-A+CA-A have the highest percentage of wound closure. *C. asiatica* contains high levels of triterpene compounds, and its wound-healing activities have been studied extensively in *in vitro* and *in vivo* model. Asiaticoside, one of the triterpene compounds isolated from this vegetable, appears to have a role in wound healing via increased type I collagen synthesis and promotion of

angiogenesis (Shukla et al., 1999a; Shukla et al., 1999b; Ruszymah et al., 2012; Azis et al., 2017). In microarray analysis, asiaticoside (30 µg/ml) was shown to influence gene expression related to cell proliferation, cell cycle processes, and the synthesis of extracellular matrix (Lu et al., 2004). Methanol fraction of *C. asiatica* extract (contain 2.4% asiaticoside) was non-toxic to human dermal fibroblast (HDF), and human dermal keratinocytes (HaCat) and both cells were able to proliferate at a concentration of 100 µg/ml and 0.19 µg/ml (Azis et al., 2017).

The current findings showed that AI-A and AI-M were able to enhance migration of fibroblast cells (*in vitro*). Based on literature review, no *in vitro* wound healing study and only several *in vivo* wound healing studies of *A. indica* were reported. Ganeshkumar et al. (2012) and Zahidin et al. (2017) reported that excision and incision wound on rats that were topically applied with 10% (w/v) of ethanolic *A. indica* extract demonstrated acceleration in wound healing (the wounds were healed in 18 days compared to the untreated rats that healed in 24 days). The wound healing activity is determined by the increased rates of wound contraction, improvement of cell proliferation, enhanced TNF-α levels during the early stage of wound healing, and enhanced expression of type I and type III collagen (Reddy et al., 2002; Laut et al., 2019).

Few studies have examined the wound healing activity of *S. grandiflora*, and our results showed that single and combined extracts of *S. grandiflora* demonstrated the lowest percentage of fibroblast migration compared to the other vegetable extracts. Sheikh et al. (2011) reported wound healing activity of an ethanolic extract of *S. grandiflora* flower using an *in vivo* model. In their study, the ethanolic extract was prepared as a 2% and 4% ointment and topically applied to rats. Both concentrations resulted in significant wound closure when compared with the control group [Nitrofurazone ointment, 0.2% (w/w)]. However, no possible mechanism was suggested

for the wound healing properties of *S. grandiflora*. Previous literature reviews also reported that both *A. indica* and *C. asiatica* have potent wound healing abilities as a single agent (Shukla et al., 1999b; Zahidin et al., 2017). However, to the best of our knowledge, the present study is the first study to report the effects on wound healing when both extracts of *A. indica* and *C. asiatica* are combined. Our results indicate that CA-A and AI-A+CA-A can actively recruit cells into the wounded area and enhance proliferation of NHDF. Even though the percentage of wound closure for single and combined vegetable extracts were almost similar, the combined extracts have the potential to be developed as an efficient therapeutic agent in comparison with the single vegetable extract. In a combined vegetables extracts, different mechanism or mode of actions-related with wound healing process was exerted by each extract and this will increase the efficacy of the combination of the vegetables in treating disease (Chou, 2006; Ncube et al., 2012; Sulekha et al., 2017).

In general, from the previous wound healing studies of *A. indica*, *C. asiatica*, and *S. grandiflora*, few mechanisms of action attributed to the wound healing activities had been reported such as accelerate the proliferation of fibroblasts and keratinocytes, stimulation of angiogenesis, prevention of prolonged inflammation stage by the increased level of enzymatic and non-enzymatic antioxidants, enhanced tensile strength, hasten wound contraction, and induced synthesis of collagen at the wound site.

5.4 Antibacterial Properties

Our data are comparable with those from a previous study reported by Ishak et al. (2013) and Zahidin et al. (2017). The authors reported that methanol extracts of *A. indica* leaves showed inhibitory activity against *S. aureus* (gram-positive) at concentration 100 mg/ml with diameter zone of inhibition of 19.00 ± 0.58 mm and minimum inhibition

concentration (MIC) value of 0.47 mg/ml. Evangeline et al. (2015) reported that methanol extract of *A. indica* exhibited inhibition towards gram-negative *E. coli* with MIC value of 1.64 µg/ml. Rajaselvam et al. (2012) also reported that aqueous extracts of *A. indica* demonstrated inhibition of *B. subtilis*, *E. coli* and *S. aureus* which showed inhibition with diameter zone of 15 mm, 12 mm, and 13 mm, respectively. However, information such as inhibition methods and the preparation and concentration of extracts used in the study was not indicated. Saranraj et al. (2010) reported that ethanol and ethyl acetate extract of *A. indica* (100 mg/ml) showed antibacterial properties against *E. coli*, *S. aureus*, *B. cereus*, and *B. subtilis* (diameter zone of inhibition: 11–30 mm).

In another study by Taemchuay et al. (2009), ethanol and water extracts of *C. asiatica* at 800 mg/ml exhibited inhibition of *S. aureus* ATCC 25923 with a zone of inhibition of 6.44 ± 0.73 mm and 17.16 ± 1.47 mm, respectively, a finding similar to this current study except for lack of inhibition by CA-A against *S. aureus*. Byakodi et al. (2018) reported that a methanol extract of *C. asiatica* was rich in phenolic content including flavonoid (13.20 µg/ml), phenols (43.70 µg/ml), tannins (30.09 µg/ml), and terpenoids (3.08 µg/ml). The extract exhibited antibacterial activities and showed a high zone of inhibition for *M. luteus* (15 mm). Soyingbe et al. (2018) reported that methanol, ethyl acetate, acetone, and water extract of *C. asiatica* exhibited antibacterial activities when tested against wide range of pathogenic bacterial such as *S. aureus* ATCC 6538, *Streptococcus agalactiae* ATCC 12386, *B. cereus* ATCC 10702, *Enterococcus avium* ATCC 14025, *E. hirae* ATCC 10541, *E. faecalis*, *E. gallinarium* ATCC 49573, *E. coli* (ATCC 25922), *P. aeruginosa* ATCC 10031, *Proteus mirabilis* ATCC 29906, *P. vulgaris* ATCC 49132, *K. pneumoniae* ATCC 13883, *Acinetobacter calcaoceuticus anitratus* (CSIR), and *S. typhi* ATCC 14028.

Our findings show that the methanol extract of *S. grandiflora* (SG-M) exhibited antibacterial activity against all evaluated gram-negative and gram-positive bacteria. A previous study showed that 500 mg/ml ethanolic extract of *S. grandiflora* produced an inhibition zone of 9.46 mm for *E. coli*, 8.91 mm for *S. aureus*, and 9.81 mm for *P. aeruginosa* (Zarkani, 2016). The result is comparable with our finding in which the methanolic extract of *S. grandiflora* at 600 mg/ml demonstrated an inhibition zone of 8.90 mm against *E. coli* and 8.6 mm against *S. aureus*. In another study, green synthesis of silver nanoparticles (20 µg/ml) from an aqueous extract of the leaves of *S. grandiflora* showed inhibition of *S. aureus* and *Salmonella enterica* with an inhibition zone of 10.54 mm and 15.67 mm, respectively (Das *et al.*, 2013).

However, the tested concentration (200–800 mg/ml) of single and combined vegetable extracts and the reported zone of inhibition varied from the previous studies, likely because of differences in the tested compounds (isolated compounds, fractions or crude of extracts) method or solvent used for extraction (Debalke et al., 2018). Our findings indicate that single and combined aqueous and methanol extracts of *A. indica*, *C. asiatica*, and *S. grandiflora* exhibit weak antibacterial properties against pathogenic bacteria that are implicated in wound infection. Any test microbial agent must have a zone of inhibition more than 17.0 mm to be considered as sensitive and potent antimicrobial properties (CLSI, 2020).

The concentrations of vegetable extracts used in this study were high when compared to the previous studies because the antibacterial effects exhibited at concentration more than 200 mg/ml. This is also in agreements with similar work which justify our range of concentrations (Odunbaku et al., 2008). The vegetable crude extracts were dark green, and solid-caramelized form and antibacterial activities were exhibited at high concentration of extracts. Because of that, the vegetable extracts and bacterial suspension

were not able to mix thoroughly. Thus, MIC and FIC for each of the single and combined vegetable extracts were not able to be determined. Several recommendations to verify the antibacterial properties of the vegetable extracts are: i) to test on the other species of bacterial strains, ii) preparation of vegetable extracts with other solvents (wide range of polarity), and iii) antibacterial studies on different fractionates of the vegetable extracts.

From our literature survey, not many antibacterial mechanistic studies had been done for *A. indica*, *C. asiatica*, and *S. grandiflora*. Only silver nanoparticle of *S. grandiflora* and triterpenoid of *C. asiatica* were extensively investigated for their antibacterial mechanisms of action. Krishnaraj et al. (2010) synthesized silver nanoparticle from aqueous extract (leaves) of *S. grandiflora*. They demonstrated antibacterial properties against waterborne pathogens such as *E. coli* and *Vibrio cholera* with MIC value of 10 μg/ml. The silver nanoparticle was able to disrupt cell membrane permeability and respiration activity (alteration of respiratory enzymes such as cytochrome oxidase, malate dehydrogenase, and succinate dehydrogenase) of bacterial cells.

5.5 Antifungal-demelanizing Properties

The green-coloured chromoprotein or black-insoluble pigment contained in the heads of conidiophores of some *Aspergillus* and *Penicillium* species belong to the group of melanin pigments (Heleno et al., 2013). Melanin can contribute to fungal virulence and survival by functioning as a 'shield' (Gómez & Nosanchuk, 2003; Rosa et al., 2010). In the current study, methanol extracts of the vegetables (single and combined) displayed a sublethal and subinhibitory minimum demelanizing concentration (MDC), indicating that they may be directly involved in the suppression or modification of the demelanization mechanism. The demelanization results reported here are of significance because the

MDC (sublethal) for the fungus was at small doses of extract compared with the inhibitory and fungicidal doses.

The current results indicate that methanol extracts of *A. indica*, *C. asiatica*, and *S. grandiflora* have potent antifungal properties in agreement with the results of previous studies. Sakthi et al. (2011) tested ethanol and ethyl acetate extract of *A. indica* (concentration range of 100–300 mg/ml) against *A. flavus, A. niger, A. fumigatus, Candida albicans, C. glabrata,* and *Penincillium chrysogenum* via disc diffusion assay. Both extracts showed inhibition against all tested fungus except *A. niger*, with diameter zone of inhibition between 8–28 mm. A crude methanol extract of *A. indica* exhibited strong antifungal property against *C. albicans* with MIC of 0.469 mg/ml (Solomon et al., 2005; Ishak et al., 2013).

Our result showed that aqueous extract of *C. asiatica* (single and combined extracts) did not have antifungal property against *A. niger*. However, according to Dash et al. (2011), at a concentration of 2000 mg/ml, the aqueous extract of *C. asiatica* exhibited antifungal activity against *A. niger* and *C. albicans* with diameter zone of inhibition of 11 mm and 9 mm, respectively. Restuati & Diningrat (2018) investigated the antifungal property of methanol extract of *C. asiatica* using agar well diffusion method. At concentration range of 100–500 mg/ml, the methanol extract showed antifungal activity against *A. niger* and *Fusarium oxysporum* with diameter zone of inhibition of 15–20 mm (MIC = 66 mg/ml) and 14–15 mm (MIC = 96 mg/ml), respectively.

From our literature survey, there was no reported antifungal study of *S. grandiflora*. Because of that, the comparison was made between sister species of the same genus. At a concentration of 0.5 mg/ml, methanol extract of *S. sesban* expressed a different degree of antifungal activity (diameter zone of inhibition) when tested against *A. fumigatus* (10.5 mm), *Colletotrichum gloeosporioides* (11.0 mm), *Cuvularia lunata* (18.75 mm),

Fusarium oxysporum (23.3 mm), and *Verticillium glaucum* (14.8 mm) (Mythili & Ravindhran, 2012). A partially purified protein, with a molecular mass of 50 kDa, isolated from seeds of *S. virgate* induced inhibition of *A. niger* (38–42%), *Clasdosporium cladosporioides* (30–35%), and *C. gleosoporioides* (12–23%) (Praxedes et al., 2011).

However, comparative analyses of antifungal activity are unavailable as few studies to date have investigated the antifungal properties of these three vegetables. Previous studies (Solomon et al., 2005; Sakthi et al., 2011) have reported that ethanol and ethyl acetate extracts of *A. indica* did not show antifungal effects towards *A. niger*. In contrast, AI-M showed inhibitory effects (MIC = 50 mg/ml), suggesting that the compound may be suitable for the treatment of aspergillosis and wound infections caused by *A. niger* (Pasqualotto & Denning, 2006). This discrepancy might be attributable to differences in the extraction method and the variety of phenolic groups and structures responsible for the numerous therapeutic effects of each plant, including antifungal activity (Harborne, 1994). To the best of our knowledge, our study is the first to report antifungal activity for combinations of *A. indica*, *C. asiatica*, and *S. grandiflora* extracts. From the data, the single AI-M extract appeared to exhibit the most potent antifungal-demelanizing activity among the evaluated extracts.

5.6 Stimulation of Nitric Oxide (NO)

The current study showed increased production of NO when RAW264.7 macrophage cells were stimulated with aqueous vegetable extracts and no significant effect following treatment with methanol vegetable extracts. The RAW264.7 and J774.2 are the *in vitro* model widely used to study inflammation with different mechanisms of action (Cabral et al., 2018). These findings are in agreement with a previous study that reported both inhibitory and stimulatory activities of ethanol and aqueous extract of *C. asiatica*,

respectively, on NO production in J774.2 mouse macrophages (Punturee et al., 2004). The infected RAW264.7 cells were able to produce more than 30-fold TNF-α (pro-inflammatory cytokines) and induce higher inflammation response compared to the J774.2 cells (Lindmark et al., 2004; El Aamri et al., 2015).

Among the three vegetables in this study, the immunomodulatory effects of *C. asiatica* have been extensively studied compared to *A. indica* and *S. grandiflora*. From the literature survey, no combination studies have been done to investigate the immunomodulatory activities of the vegetables. A previous study showed that J774.2 mouse macrophages treated with water and methanol extract of *C. asiatica* produced 1.6 μM and <0.2 μM of NO, respectively (Punturee et al., 2004). However, in the present study, RAW264.7 mouse macrophages treated with aqueous extract of *C. asiatica* increased levels of NO. NO production may vary by cell line and investigated compound/extract. However, methanol vegetable extracts stimulated low NO production compared with aqueous extracts in both studies, and NO production from RAW264.7 cells was increased following treatment with aqueous vegetable extracts.

Overall, the current finding showed that single and combined aqueous vegetable extracts demonstrated excellent NO stimulator. Single and combined methanol extract showed no significant level of NO production at low concentration (< 100 μg/ml) and significant level of NO production was observed at high concentration (> 500 μg/ml) of vegetable extracts. Thus, these results could provide formulation protocols for wound healing agents with anti-inflammatory activity (inhibit NO production) to prevent prolong of inflammation stage. Also, the formulation would be helpful to patients with immunodeficiency problems.

5.7 Antioxidant Evaluation and Protectivity against Hydroxyl Radical

The evaluation of antioxidant properties is comparable with previous studies. Marwah et al. (2007) reported that total of phenolic content of 20% aqueous ethanol extract of whole parts of *A. indica* was 72.4 mg GAE/g and it possessed good DPPH scavenging capacity with an IC_{50} value of 37.9 ± 0.1 µg/ml. Methanol and hexane extract of *A. indica* also exhibited good ABTS scavenging activity with an IC_{50} value of 6.37 mg/ml and 6.19 mg/ml, respectively (Sanseera et al., 2012). Ethanol extract of *C. asiatica* was reported to have TPC of 23.80 µg GAE/mg and FRAP reducing potential of 6.40 ± 0.04 mM Fe(II)/mg (Rattanakom & Yasurin, 2015). Aqueous extract (flower) of *S. grandiflora* also reported to have high phenolic content (162–188 mg GAE/g), DPPH radical scavenging capacity of IC_{50} = 0.08–0.21 mg/ml, and ABTS radical scavenging activity of IC_{50} = 0.12–0.23 mg/ml (Baessa et al., 2019). TPC in different calluses of *S. grandiflora* was found to range between 3.68–16.42 mg GAE/g. FRAP reducing activity and DPPH radical scavenging capacity were demonstrated to range between 101–230 mM Fe(II)/g and IC_{50} of 71.66–72.75 µg/ml, respectively (Vinothini et al., 2017).

The total phenolic content and antioxidant properties were varied when compared to the literature review because differences in the part of plant used, polarity of solvent and method of plant extraction (Alqahtani et al., 2015; Elham et al., 2015; Wu et al., 2016). The current results also demonstrated weak antioxidant activities for the combination of vegetable extracts even though they exhibited high phenolic content. This might due do the interaction between the compound(s) present in the vegetable extracts affected the efficacy of antioxidant properties (Elham et al., 2015; Sonam & Guleria, 2017). For future work, assessment of antioxidant activities at different ratio of the combination of vegetable extracts must be done to identify the best ratio that exhibited optimum bioactivities.

The current finding is in agreement with Hussin et al. (2007) which demonstrated the protective effect of *C. asiatica* extract and powder on oxidative stress (H_2O_2-induced) in Spraque Dawley rats. The rats were administered with 0.1% H_2O_2 in their drinking water for 25 weeks, and malondialdehyde (MDA) level was increased (high radical and lipid peroxidation production). In contrast, rats treated with 0.3% *C. asiatica* extract and 5.0% *C. asiatica* powder had significantly low MDA level and high catalase activity (to counter excess H_2O_2). Interestingly, they also found a significant decrease in superoxide dismutase activity of the rats, and this might be due to the antioxidant compounds in the *C. asiatica* extract and powder such as flavonoids, quercetin, catechin, and rutin. However, the method and solvent used for the preparation of the extract and powder were not mentioned. Kwon et al. (2012) showed that nano-encapsulation of aqueous extract of *C. asiatica* significantly improved the skin-protective activities against ultra-violet a (UVA) radiation by suppression of matrix metalloproteinase (MMP-1) expression, showing less cytotoxicity (< 5%) against fibroblast cells, and increased penetration into the skin cells.

Based on the literature survey, no *in vitro* study on protective effects (pre-treatment and post-treatment) of *A. indica*, *C. asiatica*, and *S. grandiflora* has been reported. Our results showed that NHDF cells pre-treated with single or combined vegetable extracts have high viability of cells compared to the post-treatment. In a different study, Annan & Houghton (2008) investigated antioxidant protective effects of aqueous extract (leaves) of *Gossypium arboretum* L. (tree cotton) and *Ficus asperifolia* Miq. (sandpaper tree) against H_2O_2. The fibroblast cells were pre-treated with the aqueous extracts and then were exposed (induced cell damage) to 100 µM of H_2O_2.

Interestingly, both extracts significantly protected the fibroblast cells at doses up to 50 µg/ml. They concluded that possible mechanism of antioxidant protection is by direct interaction of the extracts and the H_2O_2 instead of altering the cell membranes to control the cell damage and antioxidant agents with potent radical-scavenging capacity were able to facilitate wound healing (Süntar et al., 2012). This might explain the mechanism for protective effects of the single and combined vegetable extracts in the pre-treatment assay. However, further investigation must be done to verify the mechanism.

In the present study, data from TPC, FRAP, DPPH, and ABTS assays indicate that all single and combined vegetable extracts possessed good antioxidant properties (reducing and free radical scavenging capacity). Moreover, pre-treatment of NHDF cells with the single and combined vegetable extracts exhibited significant protection against H_2O_2 in comparison with post-treatment.

5.8 Analysis of Synergistic Interaction of Combined Vegetable Extracts

The primary key in folklore medicine (e.g. Traditional Chinese Medicine (TCM) and Ayurvedic medical system) is to exploit the usage of more than one medicinal plant to attain optimum therapeutic effectiveness. Each plant has established active phytochemical constituents yet, and the amount might be insufficient to achieve desirable healing effects. Because of this medicinal plants with various potency were combined, theoretically, may enhance bioactivities compared to individual plants (Parasuraman et al., 2014; Zhou et al., 2017).

Drug combination study is vital to obtain synergistic healing effect, dose, and reduction of toxicity or side effects, and provide alternative against drug resistance (Chou, 2006). Chou-Talalay theorem provides a quantitative definition for synergism (CI < 1), additive (CI = 1), and antagonism (CI > 1) in a combination of drugs (Chou, 2010). Synergism is the combination of drugs to produce greater effects than the sum of individual results. The additive is the interaction where the sum effects of a drug component equal to the effect produced by their combinations. Antagonistic interaction of combined drugs caused a reduction in total effect compared to their individual effects (Che et al., 2013).

Through the synergism analysis, we were able to determine that some vegetable extract executed excellent bioactivities independently. For example, CA-M offered the highest protectivity against H_2O_2. While the combination of CA-M with other vegetable extracts did show significant protective effects, however, no synergism interaction as indicated by the CI values (CI > 1). Another finding, synergism and antagonism can be diverse at a different level of dosages or different level of effects (Yuan et al., 2017). Depend on the physical conditions or diseases, synergism or antagonism at low/high dose or low/high effect levels may have different significance. Take anti-cancer drugs, for example, synergy at high effect level is more relevant (to kill a large fraction of cancer cells) in cancer treatment compared to low effects (Chou, 2006, 2010).

In this study, we did two types of combination, methanol-methanol (1:1) and aqueous-aqueous (1:1) vegetable extracts. Combination of aqueous vegetable extracts (AI-A+CA-A, AI-A+SG-A, and CA-A+SG-A) demonstrated synergism on DPPH free radical scavenging potential. On the other hands, the combination of methanol extracts (AI-M+CA-M, AI-M+SG-M, and CA-M+SG-M) showed antagonistic interaction. Different method and solvent used during extract preparation were able to extract

different types of phytochemical groups (Tiwari et al., 2013). This scenario reveals the complexity of interaction between phytochemical compounds that might increase (positive/synergism) or decrease (negative/antagonism) therapeutic efficacy in extract combination (Ncube et al., 2012). According to Chou (2006), determination of synergism or antagonism quantitatively takes about one to two weeks. However, it might take months or years to understand 'how' and 'why' synergism or antagonism occurs. In all, combination studies are important to investigate and to determine the best combination of vegetable extracts for the development of functional foods and therapeutic agents.

5.9 Analysis of Correlation Between Wound Healing and Other Bioactivities

In this study, we investigated the correlation between wound healing, antioxidant potential, and protectivity against H_2O_2. As discussed earlier, one of the factors that caused a delay in wound healing is prolonged inflammation in the wounded area (Guo & Dipietro, 2010). When infection occurred, neutrophils and macrophages removed pathogen that caused the infections and at the same time, they produced ROS such as hydroxyl radical in high amount to prevent further invading bacteria (Schafer & Werner, 2008; Guo & Dipietro, 2010).

According to Schafer & Werner (2008), hydroxyl radical may cause severe damage in the presence of iron of copper iron via Fenton reaction. Thus, it is vital to remove excess ROS and other radicals. Previous studies suggested several antioxidant agents detoxify the excess ROS such as glutathione, vitamin E and C, carotenoids, and phenolic compounds (Shukla et al., 1997; Rasik & Shukla, 2000). Our results showed that antioxidant-reducing activity was significantly correlated. Thus, might contribute to the wound healing activity. In general, the single and combined vegetable extracts exhibited

antioxidant-free radical scavenging and stimulation of NO activities when assessed with respective tests and assays. However, the results were not significantly correlated with the migration of fibroblasts. Thus, they might be not the main contributor to the wound healing activity.

5.10 LC/MS-MS Chemical Profiling of Vegetable Extracts

In the present study, phytochemical analysis of the vegetable extracts from *A. indica*, *C. asiatica*, and *S. grandiflora* was performed by using UHPLC system the identification of the detected compounds was made by comparing with previous reports. However, some of the identified compounds in our study were not consistent with the previous reports on similar vegetable extracts. Several factors might affect the varieties of phytochemicals present in our work in comparison with other reported works. These included factors such as geographical location (climate changes, soil conditions, or agriculture practices) and extraction methods (type of solvent, dried or fresh sample) (Govarthanan et al., 2015; Azerad, 2016).

The identified phytochemical groups in the methanol and aqueous extract of *A. indica* were comparable with previous studies. Supritha & Radha (2018) studied the phenolic compounds in the methanolic extract of *A. indica* and the identified compounds were simple phenols, catechins, anthocyanins, and glycoside forms of flavones, flavonols, isoflavones, and flavanones. Seebaluck et al. (2015) reported that the leaves of *A. indica* (the solvent used in the extract preparation was not indicated) contain acalyphin, tri-*O*-methyl ellagic acid, cyanogenic glycosides, triacetonamine, acalyphamide, and quebrachitol. In a review paper by Zahidin et al. (2017), the identified phytochemicals in *A. indica* was listed including phenolic acids (caffeic acid, ellagic acid, chebulagic acid,

ferulic acid), flavonoids (kaempferol, quercetin, and naringenin), and alkaloids (quinine). These compounds were reported to have important roles for several bioactivities such as antioxidant, anti-inflammatory, anti-microbial, and wound healing.

However, the identified phenolic compounds of *A. indica* reported in this study were not reported in any studies. Thus, the comparison was made with other plant species that reported to have similar compounds. Tiliroside (flavonoids) was identified as one of the bioactive compounds in *Pfaffia townsendii* (Brazilian ginseng) and *Malvastrum coromandelianum* (false mallow). The isolated tiliroside from *P. townsendii* and *M. coromandelianum* showed reducing and free radical scavenging antioxidant and anti-inflammatory activities (Corrêa et al., 2018; Devi & Kumar, 2020). Kaempferol-3-rutinoside isolated from *Afgekia mahidoliae* were able to accelerate the migration of keratinocyte cells and promote wound healing by activation of focal adhesion kinase (FAK) pathway (Petpiroon et al., 2015).

In this study, the major triterpenoids (asiaticoside and madecassoside) were identified in the methanol extract of *C. asiatica,* and it is agreeable with previous studies. According to Azerad (2016), there were about 40 triterpenoids (asiatic acid, asiaticoside A – G, madecassoside, madacassic acid and its derivatives) from *C. asiatica*. Besides, other compounds reported in *C. asiatica* include volatile oils, flavonoids, tannins, phenols, free amino acids, fatty acids, and sugars (Rattanakom & Yasurin, 2015; Azerad, 2016). *C. asiatica* has been widely used in commercial skincare products to reduce inflammation and prevention of acne, anti-wrinkle, and reduce scarring (Won et al., 2010; Shen et al., 2019; Venesia et al., 2020). In an *in vivo* study, a topical spray made of hydroxypropyl-β-cyclodextrin (HP-β-CD) and *C. asiatica* extract that consist of asiatic acid (0.12%), madecassic acid (0.54%), asiaticoside (0.25%), and madecassoside (1.02%) had been formulated. The spray improved wound healing of excision-wounded rats with complete

wound recovery at 14th day (Choochuay et al., 2016). Besides wound healing, asiatic acid isolated from *C. asiatica* exhibited antibacterial properties against selected bacterial pathogens by increased nucleotide leakage and bacterial membrane damage (Liu et al., 2015b).

Several groups of flavonoids and phenolic acids were identified from methanol and aqueous extracts of *S. grandiflora* in this study. Preliminary phytochemical screening of the aqueous extracts of *S. grandiflora* (leaves) detected the presence of carbohydrate, proteins, flavonoids, alkaloids, tannins, and glycosides (Reji & Alphonse, 2013). Five compounds were identified from the stem bark of *S. grandiflora* and they were 2-arylbenzofuran, sesbagrandiflorain C, 2-(3,4-dihydroxy-2-methoxyphenyl)-4-hydroxy-6-methoxybenzofuran-3-carbaldehyde, 2-(4-hydroxy-2-methoxyphenyl)-5,6-dimethoxy benzofuran-3-carboxaldehyde, sesbagrandiflorain A, and sesbagrandiflorain B (Noviany et al., 2020). Not many works reported on the phytochemical of leaves of *S. grandiflora*. Based on our literature survey, this is the first study that reported the presence of ferulic acid and *p*-coumaric acid in *S. grandiflora*. Ferulic acid and *p*-coumaric acid were ubiquitous in mushrooms, fruits (grapes, apples, tomatoes, and berries), vegetables (spinach and parsley), and whole grains (wheat, barley, and oat) (Pei et al., 2016). Ferulic acid and *p*-coumaric acid were reported to have a wide range of bioactivities such as antioxidant (Zengin et al., 2019), antimicrobial (Ibitoye & Ajiboye, 2019), anti-ageing (Shen et al., 2017), and wound healing properties (Pei et al., 2016; Zduńska et al., 2018).

In general, the therapeutic values of medicinal plants contributed by various phytochemicals such as phenolic groups, flavonoids, terpenoids, tannins, alkaloids, and polysaccharides (Supritha & Radha, 2018). Overall, the three vegetables were enriched with phytochemicals that might contribute to the bioactivities assessed in this study (migration of fibroblasts, antioxidant properties, antimicrobial activities, protectivity

against H_2O_2, and stimulation of NO production). However, further investigation must be done to determine the specific compound that is responsible for each bioactivity tested in this study.

CONCLUSIONS

6.1 Conclusions

This is the first study to investigate the synergism of extracts from three species of vegetable, *A. indica*, *C. asiatica*, and *S. grandiflora* particularly on the migration of fibroblast, antioxidant properties (phenolic content, reducing, and free radical scavenging effects), antimicrobial properties, and stimulation of NO. Our results show that the vegetable extracts demonstrated excellent bioactivities independently or in combination for each assessed bioactivity. Single vegetable extracts such as methanol extract of *C. asiatica*, methanol extract of *A. indica*, and aqueous extract of *C. asiatica* exhibited excellent protectivity effects, antimicrobial activities, and migration of fibroblast, respectively. Combined aqueous vegetable extracts *A. indica* and *C. asiatica* demonstrated synergism for migration of fibroblasts, DPPH free radical scavenging activity, and production of NO. Based on our findings, the vegetable combinations could be an advantage in the formulation development of therapeutic agents from two or more types of vegetable. In the long term, the present study helps to increase the market value of the vegetables because of the high demand in pharmaceutical, healthcare, and beauty industries. However, a thorough investigation of the mechanism of action (e.g. pharmacodynamics and pharmacokinetics) and the safety of the vegetable extracts should be performed.

6.2 Future Work

The combination of vegetables could be developed for a quad-functional treatment that has wound healing properties, antimicrobial, antioxidant, and anti-inflammatory activities. These include topical application of cream/gel to treat acute wounds and production of skincare products that use natural products as one of the main ingredients. A thorough investigation of the mechanism of action (at the molecular level) of the vegetable extracts for each bioactivity must be done including mechanism of wound healing, mechanism of protectivity against free-radical, mechanism of antimicrobial properties, and mechanism of NO modulatory. Also, an investigation of the interaction of chemical compounds (toxicology test and pharmacodynamics of drug action) in each plant that may be harmful to the human body via *in vivo* model.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

List of publication

1. **Mahmud, N.**, Wan-Mohtar, W. A., Ramasamy, S., & Manickam, S. (2020). Synergistic-antagonistic interaction of traditional herbs *Acalypha indica*, *Centella asiatica*, and *Sesbania grandiflora*: In antifungal-demelanising activities and nitric oxide immunomodulatory responses**.** *Malaysian Journal of Microbiology, 16*(5), 1-14.

2. **Mahmud, N.**, Ramasamy, S., Manickam, S., & Wan-Mohtar, W. A. (2020). Synergistic-antagonistic interaction of vegetable extracts, *Acalypha indica*, *Centella asiatica*, and *Sesbania grandiflora*: wound healing, antioxidant, protectivity, and antimicrobial properties. *Malaysian Journal of Microbiology*. *16(6)*.

3. Wan-Mohtar, W. A., **Mahmud, N**., Supramani, S., Ahmad, R., Zain, N. A. M., Hassan, N. A., Peryasamy, J., & Halim-Lim, S. A. (2018). Fruiting-body-base flour from an oyster mushroom-a waste source of antioxidative flour for developing potential functional cookies and steamed-bun. *AIMS Agriculture and Food, 3*(4), 481-492.

Paper Presented

1. **Mahmud, N.**, Ramasamy, S., Manickam, S., & Wan-Mohtar, W. A. (2018). *In vitro Wound Healing of Single and Combined Asian Vegetables*. Paper presented at the Biological Sciences Graduate Congress (BSGC), 18-20 December 2018, Chulalongkorn University, Bangkok, Thailand.