INVESTIGATION ON THE POTENTIAL OF *ARTOCARPUS ALTILIS* LATEX AS PROTEIN-BASED WOUND HEALING AGENT

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INVESTIGATION ON THE POTENTIAL OF *ARTOCARPUS ALTILIS* LATEX AS PROTEIN – BASED WOUND HEALING AGENT

by

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LIST OF ABBREVIATIONS

IC_{50}	Half-maximal inhibitory concentrations
ng	Nanogram
GO	Gene ontology
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
SD	Standard deviation
PBS	Phosphate-buffered saline
DMEM	Dulbecco's Modified Eagle Medium
Hs27	Human foreskin fibroblast line
ANNOVA	Analysis of Variance
h	Hour
MS	Mass spectrometry
MS/MS	Tandem mass spectrometry

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PENYIASATAN TENTANG POTENSI LATEKS *ARTOCARPUS ALTILIS* SEBAGAI AGEN PENYEMBUHAN LUKA BERASASKAN PROTEIN

ABSTRAK

Penyelidikan mengenai lateks tumbuhan telah dilakukan secara meluas untuk menilai potensinya dalam penyembuhan luka. *Artocarpus altilis* (buah sukun) merupakan salah satu spesies tumbuhan yang mempunyai khasiat perubatan seperti cirit-birit, sakit perut, jangkitan telinga, jangkitan kulat, kecederaan pada mata, patah tulang dan luka. Kajian ini dilakukan untuk menilai potensi lateks buah sukun dalam penyembuhan luka melalui kajian profil protein dan *in-vitro*. Sampel diambil dari Bukit Mertajam, Pulau Pinang, Malaysia. Sebanyak 106 protein dikesan melalui analisis Liquid Chromatography Mass Spectroscopy (LCMS-MS). Antaranya, 42 adalah protein berciri dan 64 protein tidak berciri. Didapati bahawa beberapa protein berciri memainkan peranan penting dalam penyembuhan luka melalui kajian literatur. Klasifikasi lanjut mengenai kehadiran protein berciri dalam Homo sapiens telah dilakukan menggunakan pangkalan data UniProtKB. Antara 42 protein berciri, 26 daripadanya ada dan 16 daripadanya tiada dalam *Homo sapiens*. Anotasi fungsi proses biologi, komponen selular dan fungsi molekul protein telah dilakukan menggunakan OmicsBox versi 3.2. Penilaian ini memberikan petunjuk lanjut tentang fungsi protein dalam penyembuhan luka. Kajian *in-vitro* telah dijalankan pada sel fibroblast Hs27. Ujian kesitotoksikan sel dinilai dalam julat kepekatan antara 0-25000 ng/ml. Nilai IC_{50} yang diperoleh untuk sampel aktif ialah 457ng/ml dan 296ng/ml untuk sampel tidak aktif masing-masing. Kepekatan 0.75ng/ml, 1.5ng/ml dan 3.0ng/ml telah dipilih untuk ujian migrasi kerana ia menunjukkan lebih daripada 70% daya maju sel. Sampel aktif menunjukkan kadar migrasi yang ketara berbanding sampel tidak aktif pada 8 jam, 12

jam dan 24 jam. Pada 24 jam, sampel aktif dengan kepekatan 0.75ng/ml menunjukkan penutupan luka 100%. Kajian ini telah memberikan maklumat tentang protein dalam *A.altilis* dan peranan pentingnya dalam penyembuhan luka.

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ABSTRACT

Research on plant latex has been done extensively to evaluate its potential in wound healing. *Artocarpus altilis* (Breadfruit) is one of the plant species with medicinal properties such as diarrhoea, stomach aches, ear infections, fungal infections, injured eyes broken bone and wound. This study was done to evaluate the potential of breadfruit's latex in wound healing through protein profiling and *in-vitro* studies. The breadfruit was collected from Bukit Mertajam, Pulau Pinang, Malaysia. A total of 106 proteins detected through LCMS-MS analysis. Among them, 42 are characterized proteins and 64 uncharacterized proteins. It is found that some of the characterized proteins plays important role in wound healing through literature studies. Further classification on the presence of the characterized proteins in *Homo sapiens* was done using UniProtKB database. Among the 42 characterized proteins, 26 of them present and 16 of them absent in *Homo sapiens*. Functional annotation of biological process, cellular component and molecular function of the proteins was done using OmicsBox version 3.2. This evaluation gives further indication on the function of the proteins in wound healing. *In-vitro* study was carried out on Hs27 fibroblast cell line. Cell cytotoxicity test was evaluated within the range of sample concentration between 0-25000 ng/ml. The IC_{50} values obtained for the active sample is 457ng/ml and 296ng/ml for inactive sample respectively. The concentration of 0.75ng/ml, 1.5ng/ml and 3.0ng/ml was chosen for migration test as it shows more than 70% of cell viability. Active sample shows significant migration rate compared to inactive sample at 8hr, 12hr and 24hr. At 24hr, active sample of 0.75ng/ml concentration showed 100% wound

closure. This study has provided information on the proteins in *A. altilis* and its significant role in wound healing.

CHAPTER 1

INTRODUCTION

1.1 Background of the Research

Artocarpus altilis (Parkinson) Fosberg, also known as the breadfruit has been used as a traditional crop by Pacific Islanders for over 3000 years (Ragone, 2006). A single breadfruit tree can yield 200–400 kg or more of fresh fruit per year (Liu et al., 2014). When harvesting breadfruit, a large amount of latex is released, particularly from the cut peduncle and from injuries on the fruit. In traditional medicine, plant lattices have been used to cure a variety of conditions, including the healing of wounds, warts, burns, ulcers, and other dermatological illnesses (Almoshari, 2022; Rosa et al., 2019). For instance, traditional healers in Tonga and Tahiti use breadfruit latex to treat wounds, rashes, abscesses, boils and sores (Han, 1998). Therapeutic effects of latex are discovered to be caused by hydrolytic enzymes like invertases, chitinases and proteases that can interfere with human physiological pathways (Upadhyay, 2011). More specifically, latex proteases make up a large fraction of the hydrolytic enzymes and are closely related to the pharmacological effects of latex (Rajesh et al., 2005). This is because majority of latex proteases exhibit procoagulant and anti- coagulant effect, which has the ability to interfere in hemostasis due to its fibrinogenolytic and fibrinolytic activities (Urs et al., 2017).

1.2 Problem statement

Although there is an abundance of *A. altilis* latex found with traditional application such as wound healing but it is not being used in any commercial applications. This might due to the lacking information on the protein profile of *A. altilis* latex which might

contribute to the wound healing. Moreover, at present there are only limited *in vitro* studies conducted to evaluate the potential of plant latex as wound healing agent.

1.3 Research objectives

This research aimed to assess the prospects of the latex of *Artocarpus altilis* as protein-based wound-healing agent. The objectives of this research were listed as follows:

1. To identify proteins from *A. altilis* latex that promote wound healing activities through *in silico* analysis.
2. To perform functional annotation of identified proteins in *A. altilis* latex.
3. To evaluate wound healing properties of *A. altilis* latex through *in vitro* analysis.

CHAPTER 2

LITERATURE REVIEW

2.1 Artocarpus altilis

There are 61 native Artocarpus plant species in Southeast Asia, Australasia, and the Indian subcontinent (Rajos-Sandoval & Acevedo-Rodriguez, 2022). The Greek words "artos," which means "bread," and "karpos," which means "seed," are where the genus name "Artocarpus" originates. The picture of *A. altilis* shown in Figure 2.1.

Figure 2.1 The picture of *A. altilis* tree

2.1.1 Origin and habitat of *Artocarpus altilis*

Humans introduced *A. altilis* to Oceania, which includes the varied islands of Melanesia, Polynesia, and Micronesia when it was first farmed in the western Pacific. It has existed for more than 3000 years as a significant staple crop (Zerega et al., 2004). Spanish explorers brought planted breadfruit trees from the Philippines to Mexico and

Central America in the sixteenth and seventeenth centuries (Parrotta, 1994). Breadfruit trees were introduced to Jamaica in 1874 after being brought from the Philippines to the French West Indies by the French adventurer Sonnerat in 1772 (Little and Skolmen, 2003). Then, these plants were dispersed across Central and South America and other Caribbean islands.

A. altilis is native to Moluccas (Indonesia), Papua New Guinea, and the Philippines (Ragone., 2018). This plant is cultivated extensively throughout the tropics and has long been a significant staple crop in Polynesia (Zerega et al., 2004; Ragone, 2006). Humid tropics, which include Asia, Oceania, Africa, Central, North, and South America, as well as the West Indies, are places where *A. altilis* is currently widespread throughout (Orwa et al., 2009). It is common among Malaysians as Buah Sukun.

2.1.2 Taxonomy of *Artocarpus altilis*

A. altilis trees are commonly planted and have become indigenous across the tropics. Humans have widely distributed both seeded and seedless varieties within and outside of their native distribution zones (Ragone, 2006). About 39 genera and 1125 species of monoecious or dioecious trees, shrubs, and rare herbs make up the Moraceae, primarily found in tropical and mild temperate climates (Haston et al., 2009). The complete taxonomy hierarchy of *A. altilis* showed in Table 2.1. The majority of plants in this family have small, unisexual, minute blooms, alternate or opposite leaves, and milky latex (Austin., 2007).

Table 2.1 Taxonomy hierarchy of *A. altilis*

Domain	Eukaryota
Kingdom	Plantae
Phylum	Spermatophyta
Subphylum	Angiospermae
Class	Dicotyledonae
Order	Urticales
Family	Moraceae
Genus	Artocarpus
Species	*Artocarpus altilis*

Plant Part	Preparation	Pharmaceutical uses	Reference
Latex	Mix equal amounts of latex from *Ficus endospermifolia* and *Artocarpus altilis* and drink	Menorrhagia	Elevitch et al., 2006
Latex	Diluted and taken internally	Diarrhea	Koch et al., 2015
Latex	Rubbed into skin	Broken bones and sciatica	Elevitch et al., 2006
Latex	Diluted and taken internally	Stomach aches	Ragone, 2018
Latex or crushed leaves	Rubbed onto skin	Skin ailments and fungal infections	Ragone, 2018
Latex or crushed leaves	Latex or juice from crushed leaves	Ear infections	Elevitch et al., 2006
Leaf bud and latex	Chew and swallow from one to five fresh buds, then drink one small glass of fresh latex. Repeat one to three times per day, until recovery is complete	Ciguatera poisoning	Ragone, 2018
Leaves	Tea made from yellowing leaves	High blood pressure, diabetes	Tamègnon et al., 2017
Latex	Fibrinolytic activity	Wound	Tamègnon et al., 2017
Leaves	The juice from the chewed or crushed leaf petiole is dripped into the afflicted eye	Injured eyes	Tamègnon et al., 2017
Bark/root	An infusion from the scraped bark or roots is sometimes taken as a potion	Urinary tract problems	Tamègnon et al., 2017

2.1.3 Pharmaceutical properties of *Artocarpus altilis*

Numerous ongoing studies are examining *Artocarpus altilis's* pharmacological properties such as injured eyes, broken bones and menorrhagia. Table 2.2 shows a list of studies conducted to evaluate the pharmaceutical properties of *Artocarpus altilis.*

Table 2.2 Pharmaceutical uses of *Artocarpus altilis*

2.2 Wound healing

An injury to the body, such as one caused by violence, an accident, or surgery, usually consists of damage to the underlying tissues and a laceration or breaking of a membrane, like the skin is called wound (Mahoney & Rozenboom, 2019). The wound has to be manage correctly to create a clean wound bed, get rid of infection, and create the best possible environment for wound healing (Karthick et al., 2010).

2.2.1 Wound healing mechanism

Even though healing is a continuous process, it can be split into four phases; hemostasis and coagulation; inflammation; proliferation and formation of scar tissue as shown in Figure 2.2 due to wound remodelling (Attinger et al., 2006). Hemostasis and coagulation occur in the wound right away after an injury. This phase aims to avoid blood loss and protects the circulatory system. This is to ensure the function of vital organs is not harmed due to injury. In addition, the hemostasis and coagulation phase functions to construct a framework for invading cells that are needed in the subsequent recovery process (Li et al., 2007). The second phase of cellular and humoral inflammatory phase are essential in forming immunological barriers against infecting germs (Hart, 2002). An early inflammatory stage activates the complement cascade that will lead to neutrophils infiltrating the wound site, whereby their primary purpose is to prevent infection (Theoret, 2016). This phase will take place 24 h to 36 h after the injury. Following this, a late inflammatory phase occurs whereby macrophages emerge in the

wound 48 h to 72 h after the injury and begin phagocytosis. Different chemo-attractive agents such as complement components, platelet factor IV, clotting factors, collagen and elastin breakdown products and cytokines will attract macrophages to the wound site. At the late inflammatory phase, lymphocytes are the final cells to arrive at the wound site, recruited by complement components, interleukin-1 (IL-1) and immunoglobulin G (IgG) breakdown products after three days after an injury (Zubin & Anire, 2006). The proliferative phase lasts from the third day until two weeks. It is defined as fibroblast movement and the build-up of the new extracellular matrix production. The remodeling phase of wound healing is essential in forming new epithelial layers and fibrous tissue (Faucher & Gibson, 2019). The duration of this stage might range from a year to two years, or even longer in rare circumstances. The primary cell type that replaces the temporary fibrin-rich matrix with a denser granulation tissue is the fibroblast. TGF-β and PDGF are two of the signalling chemicals that resident and mesenchymally derived fibroblasts respond to when they come into contact with platelets, endothelial cells, and macrophages. These signals instruct fibroblasts to either develop into myofibroblasts, which propel wound contraction, or become pro-fibrotic, laying down extracellular matrix proteins (Holly & Matthew, 2020). In conclusion, fibroblasts actually display a variety of functionalities that support wound healing in many ways.

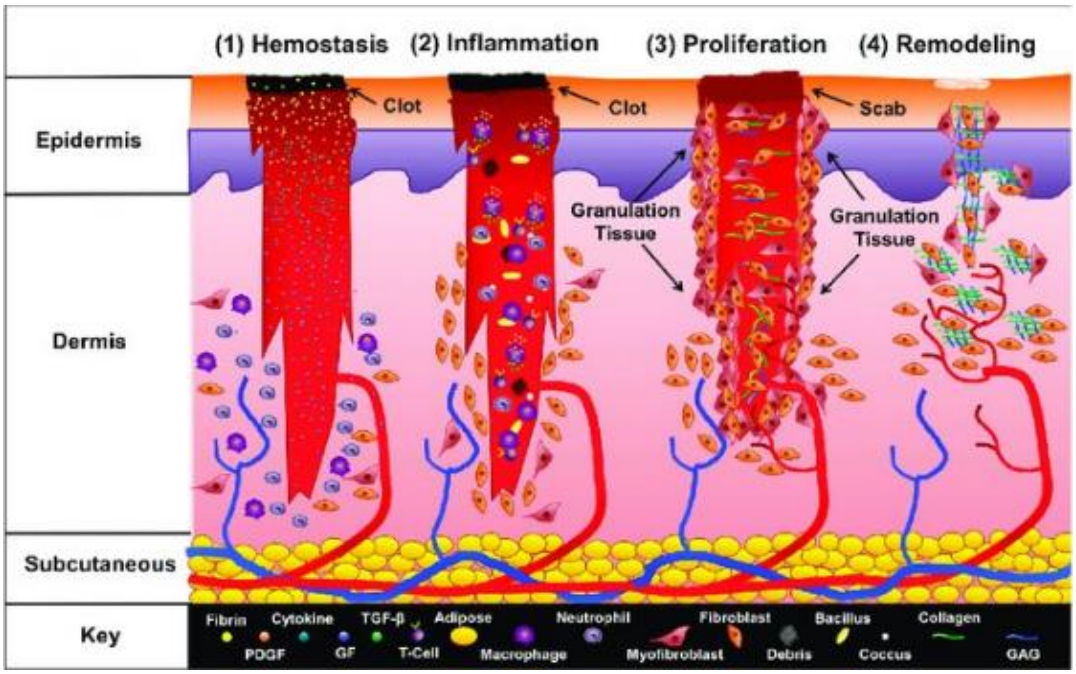

Figure 2.2 Different phases of wound healing process (Mellott et al., 2016)

2.2.2 Wound healing management and care

Cleansing, dressing, swabbing for infection, and wound debridement (in some cases) are the current standard care for injuries (Dreifke et al., 2015). The primary goals of wound healing management are quick wound healing and minimal or no impact on the function of the injured organ (Okur et al., 2020). Split-thickness autograft is the standard practice in today's wound care. This autograft technique involves removing a donor's full-thickness fascia. It will then be transplanted over the infected site. Besides that, cultured epithelial autograft (CEA) will be done by isolating target cells using a biopsy technique and expanding using 3T3 fibroblast cells in a growth medium. Moreover, tissue engineering using hyaluronic acid (HA) polymer could be one of the options because it promotes cell proliferation and growth (Dreifke et al., 2013). In addition, bio-brane dressings are appealing for use in burns. The composition of bio-brane dressings allows sera and blood within the wounded area to form a clot, which is attached to the wound bed spontaneously (Lesher et al., 2011). At the same time, the

process of epithelialization takes place. On the other hand, for the debridement of granulated tissue generated in chronic wounds, papain is frequently utilised. A number of plant lattice proteases, including *Ficus drupacea*, *Plumeria rubra* and *Jatropha curcas* have been demonstrated to have a major impact on the wound healing process (Gubbiveeranna et al., 2024).

2.3 Plant latex

Plants in the Apocyanaceae, Asclepiadaceae, Caricaceae, Euphorbiaceae, and Moraceae families generate plant latex, a viscous fluid exudate, from their laciferous tissue (Musidlak et al., 2020). Many theories have been put up on the role of latex, including defence against pathogens, defence against herbivores, protection of damaged tissue, and excretion of waste metabolites (Konno, 2011).

2.3.1 Plant latex and would healing properties

The use of plant latex for wound healing has been extensively studied. According to Rosa et al., (2019), it is hypothesized that latex can enhance wound healing by cell tissue revascularization and restoration of angiogenic function (creation of new blood vessels). *A. altilis* latex has healing properties towards glandular swellings and snakebites (Mohanty & Pradan, 2015). The identification of a serine protease that hydrolyze fibrinogen and fibrin clots lends support to the conventional application of *A. altilis* latex in wound care (Siti-Balqis et al., 2018).

Research by León et al (2020) had discovered that *Jatropha gaumeri* latex increases the cellularity of the dermis, which showed organized epithelialization for the wound healing process. Besides that, due to its potential to enhance wound healing, *J. neopauciflora* latex was effective to prevent infection of the wound, thus helping

accelerate and improve the wound healing process in animal studies using rats (Hernandez-Hernandez et al., 2021). Moreover, angiogenesis was highest in aloe vera latex, aiding in wound healing in vivo studies (Brandão et al., 2016). Animal experiments employing rabbits have demonstrated that *Calotropis procera* latex promotes the accumulation of collagen in the matrix and helps heal wounds (Aderounmua et al., 2013). Not only that, *Calotropis gigantea* latex has wound-healing capability in rats with an excision wound as the wound contraction was greatly enhanced (Saratha et al., 2009).

2.3.2 Composition of plant latex

A major component of a plant's defence mechanism against herbivory and germs is latex, a sticky emulsion made by specialized cells known as laticifers. It might be milky white or yellowish, orange to brown, or even colourless depending on the predominant composition and plant species examined. It has a wide range of active substances that benefit not only plants but also human health (Gracz-Bernaciak et al., 2021). Despite the enormous variation in latex components due to species, developmental stage, external and internal stimuli, and stresses, two main classes of physiologically active chemicals may be distinguished: proteins and secondary metabolites (Nawrot et al., 2008). Latex has a large variety of constitutive and inducible proteins, with enormous variation between various plant species. Some of examples are proteases, chitinases, oxidases, lectins, and defense-related proteins. On the other hand, secondary metabolites including phenolics, alkaloids, starch, cardenolides, and non-protein amino acids as well as proteins like compounds and rubber confers are also present in latex (Konno, 2011).

2.3.3 Proteolytic components of plant latex in wound healing activities

In addition to their protective function in plants, latices have significant pharmacological properties and are essential to the herbal treatment of wounds. In traditional medicine, they are often employed to halt bleeding and accelerate wound healing. Plant latex proteases are capable of both hydrolysing and producing clots. Haemostasis, the first stage of wound healing, depends on clot formation, but the regeneration phase's processes depend on clot hydrolysis (Urs et al., 2017). The biochemical and pharmacological characterisation of plant latex during the last 20 years has revealed their role in wound healing. Some of the past research on proteins present in plant latex and their role in wound healing presented in Table 2.2.

Table 2.3 Proteins found in plant latex and its role in wound healing

Plant name	**Protein found**	**Phases in wound healing involved**	**Process involved**	**Reference**
Carica candamarcensis	cysteine proteinase	3	Epithelisation	(Gomes et al., 2010)
Carica papaya	cysteine endopeptidases	3	Epithelisation	(Nafiu & Rahman, 2015)
Cynanchum puciflorum	cysteine proteases	1	Fibrinolytic activity	(Shivaprasad et al., 2008)
Euphorbia hirta	serine protease	1	Fibrinolyitc activity	(Patel et al., 2012)
Jatropha curcas	curcain	1	Coagulating activity	(Tinpun et al., 2020)
Pergularia extensa	cysteine proteases	1	Casienolytic activity	(Shivaprasad et al., 2010)
Synadenium grantii	glycoprotein	1	Fibrinogenolytic activity	(Badgujar, 2014)
Atrocarpus heterophyllus	serine protease	1	Fibrinolyitc and fibrinogenolytic activity	(Siritapetawee et al., 2012)
Wrightia tinctoria	serine protease	3	Epithelisation	(Yariswamy et al., 2013)

Jatropha Neopauciflora Pax	protease	4	Collagen synthesis	(Hernandez-Hernandez et al., 2021)

2.4 Proteins in wound healing

Proteins are one of the macromolecules present in all biological systems. The Greek word protos, which means "first rank of importance," is the source of the English expression "protein" (Cozzone, 2010). A hydrogen atom, a carboxyl group, an amino group, and a particular R group known as the carbon linked to a carbon atom are all present in every amino acid. The R group, sometimes referred to as the side chain, varies in size, charge, structure, and chemical composition amongst different amino acids.

2.4.1 Proteins in the human body

There are 20 distinct amino acids used to create all proteins. Those 20 amino acids can be substantially classified into 9 essential amino acids and 11 non-essential amino acids (Deschepper & deGroote, 1995). For a healthy adult, maintaining homeostasis typically takes 60 to 70g of protein per day, or around 0.8g of protein per kg of body weight (Demling, 2009). An amount of 1.5 g of protein per kilogram of body weight per day is recommended for an injured person for their wound to heal (Cohendy et al., 1999).

2.4.2 Bioactive and therapeutic proteins

Bioactive peptides have been demonstrated to have a wide range of physiological effects, such as antihypertensive, antioxidant, hypocholesterolemic effects, immunomodulatory, antibacterial, prebiotic, mineral binding and

antithrombotic (Arihara, 2006). Recently, it has been shown that proteins derived from plants and animal sources have particular bioactivities (Walther and Sieber, 2011). As bioactive proteins demonstrate tremendous advantages for human health, the market for therapeutic proteins is likewise expanding. Protein-based treatments have had significant clinical success, and their promise is now being recognized globally (Dimitrov, 2012). Based on their pharmacological activity, therapeutic proteins fit into five classes: (a) replacing a protein that is abnormal or non-functional; (b) enhancing an existing pathway; (c) developing a new activity; (d) changing a molecule; and (e) transporting compounds such as cytotoxic drugs, effector proteins, or radionuclides (Leader et al., 2008). It is hypothesised that bioactive proteins found in *Channa striata* can accelerate the healing of wounds (Kwan et al., 2019). It is frequently taken post-surgery to aid in the wound's quicker healing. In relation to this, bioactive proteins of honey such as enzymes, pollen, and other chemicals down-regulated the protease protein-encoding MMP-1 gene and up-regulated the type I collagen gene, COL1A1 for wound healing in short period of time (Ryall et al., 2022).

2.4.3 Identification of protein using a proteomics approach

Researchers have previously had difficulty identifying and understanding an organism's protein makeup. The last component of a puzzle cannot be found without a comprehensive inventory of the proteins available to an organism. Two definitions of proteomics have been discovered. The first definition of proteomics would be confining investigations utilizing only proteins to the large-scale examination of gene products (Graves and Haystead, 2002). The second would be the research of proteins using genetically-based methods, such as yeast two-hybrid analysis, genomics, and mRNA analysis (Pandey and Mann, 2000). Proteomics, a field that emerged from genomics, is

concerned with identifying and quantifying every protein in a proteome, as well as their expression, cellular connections, localizations, post-translational modifications (PTMs), and turnover with time, geography, and cell type (Zhang et al., 2013). But the principal aim of proteomics would be examining all the proteins in a cell rather than just each one individually. This objective is to get a more comprehensive and integrated understanding of biology (Graves and Haystead, 2002).

2.4.3 (a) Liquid chromatography tandem mass spectrometry (LC-MS/MS)

The best way to describe chromatography is as a physical separation process where the components to be separated are primarily dispersed between two insoluble phases: a mobile phase and a stationary phase (Niessen, 2006). Supercritical fluid chromatography, liquid chromatography and gas chromatography are among the chromatography methods that are frequently used in conjunction with mass spectrometry. There are several different mechanisms for separation found in LC and they are demonstrated in Table 2.4 (Niessen, 2006).

Table 2.4 Separation mechanisms in LC system (Niessen, 2006)

Mechanism	**Description**
Adsorption	Selective adsorption/desorption on a solid phase
Partition	Selective partition between two immiscible liquids

Ion-exchange	Differences in ion-exchange properties
Ion-pair	Formation of ion-pair and selective partition or sorption of these ion-pairs
Gel permeation/size exclusion	Differences in molecular size/, or more explicitly the ability to diffuse into and out the pore system

In the research world, reversed-phase liquid chromatography is the most often used LC technique. Molecular separation is done according to hydrophobicity. The required molecules from the mobile phase must bind hydrophobically to the immobilized water-repellent ligands coupled to the stationary phase for the separation (Aguilar, 2004). The more hydrophilic compounds can be washed first because the hydrophobic molecules in the polar mobile phase will adsorb to the hydrophobic stationary phase once the sample is brought to it. To elute the bound molecules, the mobile phase's polarity is then step by step lowered by increasing the percentage of organic solvent, often acetonitrile (Boone and Adamec, 2016). A more hydrophobic solute must be eluted with an organic solvent with a more significant concentration. Differently eluted molecules will be sent to the associated mass spectrometer for analysis. The mass spectrometer is an instrument used to quantify the mass-to-charge ratio of ionized molecules. The discovered mass in proteomics offers information about the protein's identification, chemical changes, and structure (Parker et al., 2010). The three primary parts of a mass spectrometer are an ion source, an analyzer, and a detector.

The peptides are ionized by the ion source. Ions are separated into groups by the mass-to-charge ratio (m/z) in the mass analyzer. The detector captures a signal that can be electronically intensified to produce a signal that can be measured.

2.4.3 (b) De novo sequencing and database matching

A list of proteins with corresponding scores will be created by matching each protein sequence in the database with probable fragments and their theoretical fragmentation spectrum. Following the discovery of individual peptide sequences, the collection of peptide sequences is utilized to deduce and identify the potential proteins that may have been there. The software PEAKS studio provides all-inclusive methods for discovering proteomics, including protein identification and quantification, and peptide/protein de novo sequencing (Zhang et al., 2012). PEAKS employs a unique combinatorial optimization technique to find the most appropriate sequences of peptides whose fragment ions can adequately match the peaks created in the MS/MS spectrum (Ma et al., 2003). The results from de novo sequencing are used by PEAKS software to enhance the filtration and scoring functions. Compared to existing database search tools, combining de novo sequencing with database searching dramatically improved the sensitivity and accuracy of the data provided (Zhang et al., 2012).

2.4.4 Network approach to identify protein-protein interaction in wound healing pathway

Identification of the proteins and genes involved in the recovery process and their roles in specific pathways facilitate network approach analysis (Choobdar et al., 2019). According to genomic sequencing, all eukaryotes share a significant portion of the genes defining the essential biological functions of organisms (Ashburner et al.,

2000). Gene Ontology (GO) serves as a framework for characterizing the functions of genes in any organism that has been submitted. Using the GO, three categories will be produced: cellular component, biological process and molecular part. A Bioinformatics tool known as OmicsBox can be used to search for the GO. UniProtKB database enable us to connect the proteins found with humans.

CHAPTER 3

METHODOLOGY

Table 3.1: Chemicals and equipments used for analysis

Chemicals	Brand
Ascorbic acid	HmbG Chemicals
Bradford reagent	Sigma-Aldrich
Bovine Serum Albumin (BSA)	Sigma-Aldrich
Tris-Buffer	R&M Chemicals
Tris-HCl	R&M Chemicals
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich

Equipments	Brand
Analytical balance	Sartorius
Beaker	DURAN®
Centrifuge	KUBOTA (Model: 5500, 3500)
Filter paper	Whatman®
Visible micro plate reader	Dinamica, Halo MPR-96
Microscope	Zeiss/EVO-MA10

3.1 Sample collection

The breadfruit, *Artocarpus altilis* (Parkinson) Fosberg, 16 to 18 weeks of maturity was gathered at Pulau Aman in Penang, Malaysia. The latex of the fruit collected through the incision of the epicarp of the fruit as shown in Figure 3.1 and diluted in 1:1 ratio ice-cold sodium citrate buffer (0.2 M, pH 5) containing 0.1 M sodium chloride (NaCl), 1% (w/v) ascorbic acid, 5 mM ethylenediaminetetraacetic acid (EDTA) and 1% (w/v) polyvinylpyrrolidone (PVPP). The latex was centrifuged (10,000 x g, 4°C) for 15 minutes (Kubota, Japan). The supernatant was freeze-dried for 72 h using COOLSAFE 110-4 freeze dryer at -20°C after being filtered through a Whatman No. 3 filter paper. The lyophilized material, which will be referred to as crude protein, was kept at –20ºC for further examination.

Figure 3.1 Anatomy of *A. altilis* (Siti-Balqis et al., 2018)

3.2 Soluble protein content with the Bradford method

Bradford assay was used to measure the protein content of latex from *A. altilis*, according to the Bradford method (1976), with some modifications. The protein concentration of Bovine Serum Albumin (BSA) from 0–1.0 mg/mL was prepared and used as a standard assay. Firstly, 5 µL of each protein standard and samples were loaded

into separate wells of a 96-well plate in triplicates. Bradford reagent (250 µL) was added to each well that contained standards and samples respectively. The protein samples were incubated for 45 minutes at room temperature, and the absorbance reading was measured at 595 nm using a microplate reader (Dynamica, Halo MPR-96). A standard curve of protein concentration at 595 nm was plotted as referred to Appendix B. The sample's protein concentration was determined using the equation obtained in the BSA standard curve.

3.3 Protein extraction

Protein extraction of the sample was performed in room temperature as referred to the methodologies reported by Habib et al. (2017). The C-serum fraction was added to a buffer containing 100 mmol/L EDTA, 100 mmol/L Tris (pH 8.0), 50 mmol/L Borax, 50 mmol/L vitamin C, 1% (*w/v*) polyvinylpolypyrrolidone, 1% (*v/v*) Triton X-100, 2% (*v/v*) -mercaptoethanol, and 30% (*w/v*) sucrose at a ratio of 1:5 (*w/v*). The mixtures were vortexed for 30 minutes. Following that, 2 volumes of Tris-saturated phenol (pH 8.0) were added and the mixtures were vortexed for 15 minutes. All mixtures were subsequently centrifuged for 15 minutes at 15000×g and 4°C. The upper phase was collected and precipitated with 4 volumes of cold acetone and air-dried.

3.4 LC-MS/MS analysis

The peptides were reconstituted in 100 µL of 0.1% formic acid in deionized water and filtered using the 0.45um regenerated cellulose (RC) membrane syringe filter. Mass spectrometry analysis (MS) has been performed using the LTQOrbitrap Velos Pro mass spectrometer (Thermo Fisher Scientific, CA, USA) coupled with Easy-nLC II nano-liquid chromatography system. The eluent was then sprayed into the mass spectrometer at 2.1 kV (source voltage) at 220°C. Complete scan mass analysis is done

from m/z 300-2,000 at resolving power of 60,000 (at m/z 400, FWHM; 1-s acquisition) with data-dependent MS/MS analyses (ITMS) which triggered by the 8 most abundant ions from the parent mass list of predicted peptides with the rejection of singly or unassigned charge state. Collision-induced dissociation (CID) was then applied as the fragmentation technique.

3.5 Protein identification

The data was obtained on the peptides was proceeded for an analysis in PEAKS Studio Version 7.5 (Bioinformatics Solution, Waterloo, Canada) software for de novo sequencing and database matching. UniProtKB database was used to identify the proteins found in *Homo sapiens.* Parameters for analyzing the samples have been set according to Kwan et al. (2019) with minor changes.

3.6 Bioinformatics analysis

The list of annotated protein sequences was imported into OmicsBox Version 3.2 database to perform Gene Ontology (GO) annotation of the biological process, cellular component and molecular function of the proteins.

3.7 Cell types and culture conditions

Hs27 fibroblast cells were used as they are one of the crucial cells in wound healing. The cells were maintained in correct conditions for cell cytotoxicity and cell migration study.

3.7.1 Cell types and media

Hs27 cell lines were purchased to be used in this research and were maintained in DMEM. Hs27 is a fibroblast cell that was isolated from the foreskin of a Black male

donor. All media were prepared with 5% heat-inactivated fetal bovine serum and 1% penicillin (Dahham et al., 2015).

3.7.2 Routine cell feeding and maintenance

Hs27 cells were preserved at 37°C in an incubator that had been sterilized and humidified with 5% CO_2. To ensure the cells were healthy and free from contamination, cell morphology and media color and turbidity of the media were carefully observed.

3.7.3 Subculture of cells

Subculture of Hs27 cells was carried out when the flask was confluent. The process was conducted in a biosafety cabinet Level 2. The process began with eliminating old media from the flask, then adding 2/3 mL of phosphate buffer saline (PBS) to wash the cells. The PBS was discarded after mild shaking of the flask. Next, 1000 µL of 1× trypsin-EDTA solution was introduced into the flask and kept in the incubator for 5 minutes to detach the cells. Later, 5 mL of DMEM was added to stop the tryptic reaction. The cells were then subcultured into new flasks at a ratio of 1:3.

3.7.4 Cell counting

Using a hemacytometer, the number of Hs27 cells was counted, as reported by Macleod and Langdon (2004). 10× magnification was used to observe the cells using the microscope. 10 µL of single-cell suspension was loaded to the groove area of the slide, then the total number of cells in the four outer chambers was averaged. The following formula determines the total number of cells per 1ml of media:

$$total\ number\ of\ cells\ from\ four\ chambers/4 \times 10{,}000$$

This factor is equal to the volume of each square as the length and the depth of the square are 1 mm and 0.1 mm, respectively.

3.8 CCK8 Assay Kit for cell cytotoxicity

The Hs27 cells were distributed into 96 wells. It was then incubated overnight at 37°C and 5% Co_2. Sample of different concentrations ranging from 0-25000ng/ml (100µl) was added following the incubation. After 72h of incubation, 20µl of CCK8 (Dojindo, Japan) was added, and incubated for 4 h. ELISA readings were taken at 595nm after incubation.

3.9 Half-maximal inhibitory concentration (IC_{50}) for cell cytotoxicity

The IC_{50} value for each sample, which is the concentration required to inhibit the biological process by 50%, was determined by plotting percentage cell viability against the concentrations of the samples.

3.10 Scratch assay for cell migration activity

Scratch assay was done as referred to the methodologies described by Ponnusamy et al., (2015). The cells were distributed into 24 wells. The cells were injured by being scratched using yellow tips. The contaminants were subsequently removed from the wells by washing them with 1000µl of PBS. 1000µl sample of three non-toxic concentrations was added. As for the control 1000µl of media was added. Cell migration was photographed at 0 h, 8 h, 12 h and 24 h intervals. Using ImageJ imaging software, data were further analysed to quantitatively determine the depth to which the cells penetrated the wound. The percentage of wound closure at each time point was calculated as equation;

% wound closure= $\frac{(Do-Dn)}{Do}\times 100\%$

where Do is the distance between the scratch's two sides at the beginning, and Dn is the distance at the time the measurement was made.