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“Phlorotannin from algae for diabetic wound healing plasters”

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Abbreviation

Phlorotannin	**PH**
Phlorotannin rich extract	**PH-rich extarct**
Saccharina latissima	**S. *latissimi*** **Saccharina**
Alaria esculenta	**A.esculenta** **Alaria**
Ultra violet	**UV**
Extracellular matrix	**ECM**
Crude extract	**CR**
Pure Extract	**PE**
Ultrasound-Assisted-Extraction	**UAE**
Total Phenolic Content	**TPC**
Normal Human Dermal Fibroblast	**NHDF**

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Abstract

Background: One of the major health concerns for people with diabetes is Wounds. Phlorotannin (PH), one of the natural compounds extracted from brown algae (seaweeds), has been demonstrated in previous studies to have several biological effects, including stimulation of cellular proliferation. Accordingly, the present study was designed to create a novel approach for investigating the influence of PH on (NHDF cell proliferation for wound healing applications, particularly diabetic wound healing plasters. This study represents the first study on the application of wound healing with crude and pure extracts of phlorotannins, obtained from brown algae.

Methods: In this study, firstly, we conducted ultrasonic-assisted extraction techniques with 80% ethanol to extract and purify the PH-rich extract (polyphenol) from brown algae (Saccharina latissimi and Alaria esculenta). Then our work was to highlight the in vitro healing potential of a PH-rich extract from brown algae (*S.latissimi* and *A.esculenta*) using the MTT assay on normal human dermal fibroblasts (NHDF). The ability to stimulate cell proliferation under the influence of different concentrations of PH-rich extract (ranging between 25 and 200 mg/uL) was determined compared to the control (an untreated in vitro-simulated wound).

Result: Our results showed that the treatment of NHDF with the crude PH-rich extract from *S.latissimi* at a concentration of 100 mg/uL increased cell proliferation and viability, compared to the control group. In contrast, treatments with a PH-rich extract from *A.esculenta* decreased cell proliferation and viability, compared with the control. In addition, we have shown that crude PH-rich S. latissimi extract contained 10.3 ± 2.2 mg GAE/g (total phenolic or PH-rich extract were expressed in milligrams of gallic acid equivalents per gramme), and the pure PH-rich extract contained 13.4 ± 7.6 mg GAE/g. In contrast, the crude PH-rich A. esculenta extract contained 37 ± 4.4 mg GAE/g and the pure PH-rich extract contained 40.2 ± 6.6 mg GAE/g. *Alaria esculenta* shows that it contains a considerable amount of polyphenols compared to *Saccharina latissima*'s PH-rich extract.

Conclusion: This study shows that *Saccharina latissima*, a source of the phlorotannin compound, increases cell viability and proliferation and therefore has wound healing potential.

Introduction

1.1 The Skin : The Structure and Function of the Skin

The skin is considered the greatest organ in the human body (Jin et al., 2013). Skin is the crucial barrier that supports the body and protects against the external invasion of pathogens, extreme water loss, and UV rays, as well as aiding in maintaining a normal body surface temperature (Rhim et al., 2010). The skin's system is comprised of three functional levels that are themselves tiered. The epidermis provides the primary protective role; the dermis includes a network of capillaries that are important for delivering nutrients and eliminating harmful waste; the membrane of the basement connective tissue between the epidermis and dermis and acts as an adhesive junction (Jones et al., 2002, Nishiyama et al., 2000). Despite the fact that these layers are distinct, they never operate independently. Their various biochemical and physiochemical properties cause the skin to function as a single organ (Nishiyama et al., 2000).

1.1.1 Epidermis

The epidermis prevents excessive water gain or loss and serves as the first line of defence against chemical and physical aggression. This layer is composed of both vascular and stratified squamous epithelium. Up to 95% of the epidermis consists of keratinocytes, but it also contains Langerhans cells, which are responsible for immune response, melanocytes, which are responsible for pigmentation, and Merkel cells, which are responsible for nerve conduction (Eichman, 1990).

1.1.2 Dermis

The dermis is mainly composed of fibroblasts, connective tissue, a few number of adipocytes, and macrophages, which are mainly responsible for the skin's structural integrity. Fibroblasts are the primary cells of the dermis. They play a vital role in wound healing, generate proteins for the extracellular matrix that connects the dermis and epidermis, and form and maintain connective tissue. Collagen is the primary connective tissue fibre, but elastin is also present (Eichmann, 1990). The dermis consists of a papillary layer and a reticular layer. Despite the absence of epidermal tissue layers, the dermis is intricate and capable of physical interaction with the epidermis (Gartner and Hiatt, 2011).

1.1.3 Layer of Basement Membrane

The basement membrane layer is located between the epidermis and the dermis (epidermal-cutaneous junction). This junction controls the skin's structural integrity by providing adhesion and a dynamic connection between two distinct cellular compartments. This layer of basement membrane controls the migration of molecules between the dermis and epidermis depending on their charge and size, hence keeping the appropriate composition of the dermis and epidermis (Timpl, 1996).

1.2 Biology of Wound healing

The wound of the skin has been defined as a structural and functional disturbances of the epidermis, dermis, and basement membrane layers, that can be result of microbiological, thermal, physical, chemical and immune tissue injury (Kim et al., 1998; Maffer et al., 2015). Hence, itself requires powerful and efficient healing processes following damage. Cutaneous wound healing is the process of restoration the stability and physiological function of these tissues (Ghahary and Ghaffari, 2007; Kim et al., 1998). This wound healing mechanism consist of four phases that overlap: Coagulation (haemostasis), inflammation, proliferation (granulation), and remodelling (maturation). Each stage is categorized by infiltration into a specific cell type in the wound site (Diegelmann, 2004; Eming et al., 2014) and all of them interact with another through chemical signals to maximize reparation (Öztürk et al., 2012).

As shown in Figure 1, in the first phase, coagulation occurs immediately after wounding, in order to prevent additional tissue loss due to blood vessel damage. It is characterized by the rapid formation of a fibrin clot to reestablish coagulation. (Arya et al.,2014; Golebiewska & Poole, 2015). Platelets in the blood initiate the clotting cascade and wound healing by secreting several growth factors. Fibrin breakdown products and platelet-derived growth factors act as inflammatory cell attractants towards the end of the haemostasis phase (this can take up to five days, depending on the therapy) (Enoch & Leaper, 2008). In the following phase, called inflammation, neutrophils migrate to the wound site, ingest foreign detritus, phagocytose bacteria, and release proteolytic enzymes (Enoch & Leaper, 2008; Martin & Leibovich, 2005). Blood monocytes reach the wound site and develop in to the macrophages, release proteases to remove wound debris (Enoch & Leaper, 2008). Blood monocytes also release a combination of bioactive chemicals, such as transforming growth factor- 1 (TGF-1), which promotes the migration of fibroblasts and epithelial cells (Delavary et al., 2011). Blood monocytes also release a variety of bioactive chemicals, including transforming factor-1 (TGF-1), which promotes the fibroblast and epithelial cells migration.

The phase of proliferation usually starts nearly three days after injury and consists of a variety of activities such as, angiogenesis by endothelial cells, development of granulation tissue by fibroblasts, and re-epithelialization by keratinocytes (Fan et al., 2012; Kasuya and Tokura, 2014). During this stage, fibroblasts create a large amount of extracellular matrix (ECM), especially collagen, which replaces injured tissue with granulation tissue. Keratinocytes, on the other hand, proliferate and form the functional epidermis during re-epithelialization to close the wound and protect the underlying tissue from further damage (Fan et al., 2012). When wound healing occurs, a population of dermal fibroblasts actively remodels the disordered ECM characteristic of granulation tissue (Fan et al., 2012), whose numbers are progressively reduced by apoptosis (Akasaka et al., 2010). Scar tissue (also known as fibrosis) is the consequence of wound recovery, with fibroblasts scattered throughout the collagen-rich ECM. Compared

to the original tissue, the scar tissue is distinguished by its diminished biomechanical activity, unique texture, and functional properties (Akasaka et al., 2010).

The wound categorization depend on their cause, symptoms, the depth of wound, location, kind of damage, and the appearance of clinical (Sabale et al., 2012). On the basis of the physiology of wound healing, a skin wound can be classified as either acute or chronic (Zeng et al., 2018). The acute wounds are identified as those that show symptoms of healing within three months of damage and move through the normal stages of healing (Walker et al., 2015). Typical causes of acute wounds involve accidents, lacerations, surgery, burns, tissue damage, and abrasions, which recover in a reasonable time (Wiegand et al., 2009; Zeng et al., 2018).

On the other hand, various local disturbances and systemic illnesses can delay the healing of wounds (Eming et al., 2002). The consequent prolonged inflammation and the toxic microenvironment are the primary causes of the shift in wound condition from acute to chronic. The most prevalent chronic wounds include venous leg ulcer, pressure ulcer and diabetic foot ulcers (Cullen et al., 2002).

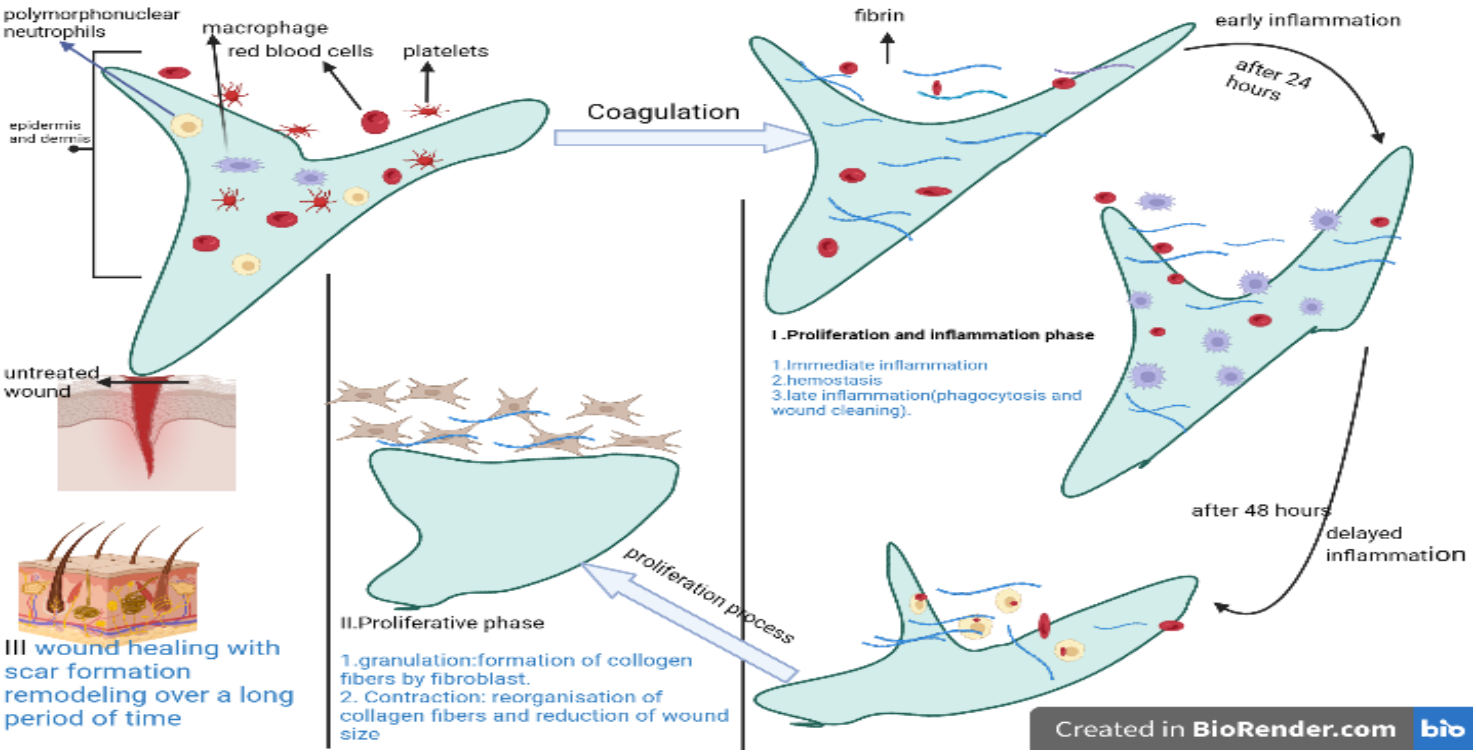


Figure 1 : *The wound healing phases and the effects of fibroblasts in this mechanism. Created with BioRender.com*

1.3 The Chronic Wound

A chronic wound is described as one that takes longer than three months to heal or fails to heal ordinarily. Wounds with healing issues are divided into four classes: pressure, atrial insufficiency ulcer, venous, and diabetic (Hart et al., 2002). This study will exclusively focus on diabetic ulcers.

Diabetes is a serious condition and among the most prevalent chronic diseases worldwide. It impacts around 381 million adults worldwide (Patel et al., 2019), and an estimated 175 million cases remain undiagnosed (Patel et al., 2019). In the United States, for example, 11 million people were detected with the condition in 2001, representing around 4% of the population; this number is expected to increase to 29 million in 2050 (Lipsky et al., 2004). According to the International Diabetes Federation (IDF), these statistics will continue to rise in the future (Lipsky et al., 2004). Type two diabetics is the most common and approximately 85–90% of diabetics suffer from type 2 diabetes, while only 10–15% have type 1 diabetes (Herman & Herman, 2022).

Diabetes mellitus is a chronic condition related to the endocrine system and insulin insufficiency. Diabetes mellitus presents as insulin resistance and hyperinsulinemia, which can evolve into insulin deficiency (Gillespie, 2006; Scheen, 2003). If not appropriately treated, this insulin resistance can cause hyperglycemia. Prolonged periods of hyperglycaemia cause organs and tissues in the body to become damaged and dysfunctional, which can result in neuropathy, nephropathy, hypertension, retinopathy, loss of sensation in the feet, an increased chance of heart attacks, and cardiovascular disease (Alam et al., 2014; Brownlee, 2005). Kalan et al. presented that the effects of delaying wound healing in diabetes patients are the most expensive and dangerous, such as gangrene, sepsis, bacterial infections, amputations, and even death (Kalan et al., 2019).

Factors influencing wound healing can be classified as systemic and local. Systemic factors are always present in the individual, as demonstrated in disorders such as diabetes, stress, obesity, and ageing, whereas local factors involve infection, oxygenation, and foreign substances that have a direct effect on the wound locally (Guo & DiPietro, 2010). Local factors cause elevated amounts of proinflammatory cytokines and reactive oxygen species (ROS), decreased cell and protease function, and a lack of growth hormones, among others (Frykberg & Banks, 2015); see Figure 2.

Type 2 diabetes is assumed to be among the most important systemic causes for wounds to take longer to heal, and has been demonstrated to cause persistently increased pro-inflammatory cytokine levels (Chen et al., 2015). It has been shown that a long inflammatory stage slows wound healing due to less growth factor activity, less oxygen in the area, infections, and more proteases (Demidova-Rice et al., 2012). Insufficiency of immune cells has been determined in diabetic patients as a result of decreasing growth factors and

reduced antimicrobial activity. All of these factors mean that chronic wounds are easily infected, especially by bacteria, and function as access points for systemic infection. Aerobic (facultative) pathogens, including *Pseudomonas streptococci* and *Staphylococcus aureus*, are the leading causes of delayed healing and infection in acute and chronic wounds (Lipsky et al., 2004; Louie, 1976).

In addition, diabetics have high levels of ROS, which destroy the ECM (Schultz et al., 2005), increase protease levels, and inhibit growth factors (Lauer et al., 2000). Degradation of growth factors is also a significant contributor to delaying the healing of the wounds (Demidova-Rice et al., 2012). It has also been shown that keratinocytes from unhealthy skin generate fewer growth factors and have a lower migratory potential than keratinocytes from healthy skin (Usui et al., 2008). Similarly, chronic wound fibroblasts have poor sensitivity to hypoxia and reduce potential migration (Lauer et al., 2000).

Previous investigations of *in vitro* wound healing effectors focused mostly on dermal fibroblasts, dermal endothelial cells, and epidermal keratocytes (Gottrup et al., 2000; Ud-Din & Bayat, 2017). The epidermis of the human body is made up of several layers of keratinocytes that are continuously restored to start replacing damaged or deteriorated cells. Keratinocytes and fibroblasts are the principle components of the wound-healing process (Stefanowicz-Hajduk et al., 2016). During the mid- and late-stages of wound healing, fibroblasts and keratinocytes dominate cellular interactions, gradually shifting the microenvironment from the inflammation phase to a synthesis-driven granulation phase (Werner et al., 2007). The proliferation and migration of keratinocytes are the most important steps in re-epithelialization (Wang et al., 2012), whereas fibroblasts in the location of the wound secrete extracellular proteins that enhance the rapid proliferation of keratinocytes (McDougall et al., 2006). The investigation was therefore interested in NHDF's ability to simulate wound healing in vitro.

This study focuses on the effects of a PH-rich extract of brown algae (Saccharina latissima and Alaria esculenta) on normal human dermal fibroblasts (NHDF) as a possible therapy for wound healing, which would be of interest to diabetics due to their impaired wound healing. NHDF is a key mediator during wound healing whose migration and proliferation are critical and rate-limiting steps to repair wounds due to its central role in granulation tissue formation. Granulation tissue formation is essential for healing the wounds, and its dysfunction can significantly delay or even stop the mechanism of healing (Shi et al., 2021; Singampalli et al., 2020).

Particularly, this study focused on the dermal fibroblast, which is critical for maintaining healthy skin and is controlled by cells called dermal fibroblasts, which have the capability to make collagen (Moreno-Romero et al., 2019). Furthermore, fibroblasts play an essential role in conserving the microbiota and the immunity of the skin: production of interferon, stimulation and production of the interleukins IL-6, IL-8,

and IL-1, and phagocytosis of aggregate collagen (Tracy et al., 2016). Fibroblasts provide an unfavourable habitat for bacteria by producing acidic sweat (Wu et al., 2017). Dermal fibroblasts are also responsible for differentiating various types of cells, such as reticular and papillary fibroblasts and intradermal adipocytes, and making extracellular matrices (Cole et al., 2018).

1.4 Novelty (originality)

Wounded skin heals more slowly in diabetic patients because wound healing, which includes a series of complex phases (inflammation, cell proliferation, matrix deposition, and tissue remodelling), cannot be completed when any of these processes are impaired. Following these issues, current wound healing treatments rely on topical dressings to provide the moist environment essential for granulation tissue and epithelialization. They also help to reduce infection risk, speed wound healing, and reduce scarring. For diabetic patients, the optimum dressing should promote quick healing at a low cost. To treat and repair wounds, this study will investigate a phlorotannin-rich extract (polyphenol) from brown algae (*Saccharina latissima* and *Alaria esculenta*). It will examine *in vitro* the ability of this phlorotannin-rich extract to increase proliferation and migration of NHDF to recover delayed wound healing before applying it as a wound dressing (plasters) for diabetic people who are suffering from delayed wound healing.

1.5 Brown Algae and Phlorotannin

Products of natural compounds are secondary metabolites produced by plants, algae, fungi, cyanobacteria, and marine animals. They have biologically important activities that can be used to treat humans or animals (Saurav et al., 2017). Frequently, natural products serve as the major compounds in drug production (König et al., 2005). The study of compound characterization and pharmacological activity based on phytochemical analysis by extraction, isolation, and fractionation of constituents (Kubelka, 1994).

Brown algae is known to contain high amounts of bioactive components, such as minerals, polysaccharides, pigments, and polyphenols. Brown algae is traditionally used as medicine and food, mostly in Asian countries (Ahn et al., 2008). Antioxidant compounds are of interest due to their advantages for human health in comparison to synthetic antioxidants, which can induce major and significant side effects (Li et al., 2012). Polyphenols were suggested as one of the most interesting compounds by researchers based on their phlorotannin antioxidant activities and ability to scavenge free radicals (Huang et al., 2005; Stanisavljevi et al., 2009). In addition, multiple studies have demonstrated their antibacterial action, making them a viable alternative to chemical preservatives (Oliveira et al., 2009; Stanisavljevi et al., 2009).

Particularly, phlorotannins (PHs), a type of polyphenol, are secondary metabolites of marine brown algae (*Phaeophyceae*) with many molecular sizes and functionalities. PH are present in cytoplasmic vesicles (Physodes) and compose the cell wall of the algae. These substances can account for more than 20% of the weight of dry algae (Ragan & Glombitza, 1986). Physodes range in diameter from 0.1 to 10 mm (Taylor, 1936; Döpfner et al., 1990; Schoenwaelder & Clayton, 1998). Schoenwaelder (2002a) evaluated the prevalence and function of physodes in brown algae. Several roles have been hypothesised for PH in the adhesion and settling of freshly fertilised zygotes in fucalean species (Schoenwaelder & Clayton, 1998b). Additionally, Roleda et al. suggested that the PH has a protective role on brown algae,including spores (Roleda et al., 2006; Wiencke et al., 2000), against environmental stresses such as UV radiation (Pavia et al., 1997; Pavia & Brock, 2000; Schoenwaelder, 2002).

According to the bioactive compounds found in brown algae, PH are recognised as having several biological effects, such as anti-inflammatory (Jung et al., 2009), anti-oxidative (Heo et al., 2009), and anti-microbial activities against bacteria associated with skin disease. Such as *Propionibacterium acnes* (Lee et al., 2014), *Staphylococcus epidermidis* (Lopes et al., 2012), and *Staphylococcus aureus* (Eom et al.,2012).Electrospun PCL/phlorotannin has been shown to promote osteoblast proliferation for bone tissue regeneration (Kim & Kim, 2012). Despite the potential benefits of utilising PH as a biological substance, no research to date has reported on its use in wound healing biomedical applications.

1.6 Purposes of Research

The purpose of this study was to investigate a phlorotannin-rich extract (polyphenol) from brown algae (*S. latissima* and *A. esculenta*) with regard to its potential for wound healing based on its corresponding antioxidant, regenerative, anti-inflammatory, and antimicrobial activity, which was detected in the previous studies.

1.7 Research Question

Do the phlorotannin-rich extracts of selected algae (seaweeds), called *S. latissimi* and *A. esculenta*, improve the proliferation and migration of normal human dermal fibroblast (NHDF) cells?

1.8 Hypothesis

I hypothesised that a phlorotannin-rich extract (polyphenol) isolated from brown algae (*S. latissimi* and *A. esculenta*) would improve wound healing by increasing NHDF proliferation and migration. Four specific aims were developed to evaluate the hypothesis, as follows:

1.9 Research Objectives and Aims:

The aim of this study was to investigate the effect of a phlorotannin-rich extract (polyphenol) from brown algae (*S. latissima* and *A. esculenta*) on NHFD proliferation and migration. Therefore, the defined objectives were as follows:

Objective One: Isolate PH-rich extract (polyphenol) as a crude extract from *S. latissima* and *A. esculenta*.

Objective two: Purify the crude extract of PH-rich extract (polyphenol).

Objective three: Determine the total PH-rich extract (polyphenol) of the crude and pure extracts.

Objective four: Examine the functional response of the cells (NHDF) of human skin to the crude and pure extracts of phlorotannin-rich extract (polyphenol) from *S. latissima* and *A. esculenta*.

1.10 Importance of Research

This study examined a phlorotannin-rich extract (polyphenol) from brown algae (*S. latissima* and *A. esculenta*) with regard to its potential for wound healing based on its corresponding antioxidant, regenerative, anti-inflammatory, and antimicrobial activities, as determined in the literature. This was addressed via experiments that checked the NHDF cells' viability. The findings of this study may lead to the development of novel wound healing plasters and solve problems with treating and managing chronic wounds. This could help reduce the financial load on the systems of health care.

1.11 Ethics

The management and use of biospecimens from the cell line of NHDF were accorded by the Coventry University ethical committee on July 7, 2022 (end date was May 13, 2023), with approval number P136517.

Materials and Methods

2.1 Extraction of polyphenols from *S. latissimi* and *A. esculenta*

Extraction is the first step in the scientific investigation of plant and algae medicines. The extraction of the chemical compound entity considered to be responsible for a given effect is a basic component of the screening technique used in bioprospecting for novel drugs. In order to isolate particular groups of chemicals from raw plant or algae material, a number of solvents and procedures are employed. However, isolating the entity of a single chemical is of little benefit unless it can be shown that it is entirely responsible for the claimed effect. According to one school of thought, the efficiency of plant or algae medicine is the result of the interaction of the numerous chemical components present, and isolating specific components diminishes the overall efficacy.

The restoration of phytochemicals is extremely technique-dependent (Dirar et al., 2019). Traditional techniques for the extraction of bioactive compounds include infusion, filtration, maceration, distillation, and expression, although microwave extraction, ultrasonic-assisted extraction, and supercritical fluid extraction are more recent techniques (Dirar et al., 2019). For example, phenolic compounds (such as glycosides and saponins) are more easily extracted using polar solvents such as ethanol and methanol, whereas non-polar solvents such as dichloromethane, ethyl acetate, and hexane produce primarily fatty acids and steroids. However, the selection of extraction approaches and the choice of solvent are governed by the desired pharmacological activity rather than the compositions of the components (Yalavarthi & Thiruvengadarajan, 2013; Dirar et al., 2019). However, the selection of extraction approaches and the choice of solvent depend more on the pharmacological activity than the combination of components (Yalavarthi & Thiruvengadarajan, 2013; Dirar et al., 2019).

In this study, an attempt was made to isolate a PH-rich extract (polyphenol) from brown algae (*S. latissimi* and *A. esculenta*), and to evaluate *in vitro* the ability of PH to increase proliferation and migration of NHDF to recovered delay wound healing, particularly in diabetic patients. Since the method and type of solvent employed in extraction are important for human health, ethanol was utilised for solvent extraction with an ultrasonic-assisted extraction (UAE) methodology devised by Kim and Lee (2002). The crude extracts (CE) were purified and fractionated using ethyl acetate and chloroform to produce the pure extract (PE), using a technique adopted from Queires et al. (2013). UAE is an alternate extraction method that generates ultrasound waves using mechanical energy (Jia et al., 2011). UAE has many advantages over conventional extraction techniques, including high efficiency, selectivity, improved quality, low energy consumption, productivity, decreased time of extraction and solvent consumption, environmental friendliness, low cost,

a high level of automation, and decreased physical and chemical hazards (Feresin et al., 2016; Kim et al., 2013).

2.2 Materials

2.2.1 Chemicals and Reagents

Ethanol (SIGMA-ALOAICH/UK), Dichloromethane (Honeywell/UK), Ethyl Acetate, Folin-Ciocateu Phenol Reagent (SIGMA-ALOAICH/UK), Sodium Carbonate (SIGMA-ALOAICH/UK), and Gallic Acid (SIGMA-ALOAICH/UK) were utilised. All reagents and chemicals used in this investigation were of analytical grade, and all of the experiments were conducted with deionized water.

2.2.2 Ultrasonic Water Bath

Ultrasonic baths (XUBA1/UK) (230 volts, 50–60 Hertz, 50 Watts) produce ultrasonic waves in tanked water to obtain full extraction of polyphenol (PH-rich extract) from algae samples with high yield in a very short extraction period. This technique of extraction is cost- and time-efficient, and it produces a high amount of extracts that are used in food supplements and medications due to the procedure's efficiency (Antes et al., 2017).

2.2.3 Chilled Buchner Funnel with Vacuum Suction

Buchner funnels employ vacuum suction for filtering when paper filters or membranes cannot survive the filtrate's chemical assault. This technique was utilised to filter the combination of 80% aqueous ethanol and a powdered algae sample.

2.2.4 Rotary Evaporator

Rotary evaporators (BUCHI, Germany) (200-230V,50/60 HZ) are used to efficiently remove solvents through the evaporation process. Their optimal extraction and distillation performance make rotary evaporators one of the most important methods used for evaporation. In this investigation, a rotary evaporator was used to remove the solvent, 80% aqueous ethanol, from the mixture.

2.2.5 Spectrophotometer

A spectrophotometer (Biochrom/USA) is an instrument used to measure the concentration of a chemical substance depending on the intensity of light absorbed after passing through the sample solution.

2.3 Methodology

2.3.1 Algae Preparation

The samples of brown algae with identification (approximately 7 gram each) were provided by the Culture Collection of Algae and Protozoa company (CCAP), SAMS Limited, Oban, Argyll, PA37 1QA, UK. These samples were used to derive a PH-rich extract (polyphenol), which was then used to test wound healing in NHDFs *in vitro*. The samples comprised *A. esculenta* and *S. latissima*, which were harvested on April 30, 2020. The samples of brown algae were washed by deionized water and stored frozen at –20 °C until used for extraction. Then, the algae were ground into a fine powder using a grinder (name of grinder). After that, the algae samples were submitted to extraction and purification, as shown in Figure 2.

2.3.2 Freeze Drying

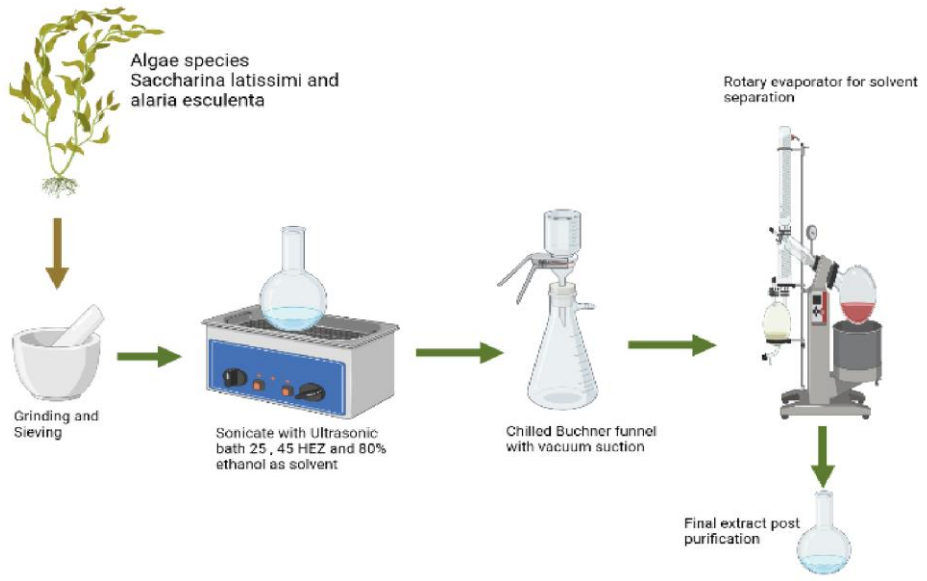
Normally, before extraction, plant samples are handled by grinding and homogenization, which may be preceded by freeze-drying or air-drying. In general, freeze-drying preserves more phenolics in plant samples than air-drying (Abascal et al., 2005). According to Asami et al. (2003), freeze-drying strawberries, corn, and marionberries yielded a higher total phenolic content than air-drying. Given the purpose of this study, the freeze-drying method was used for algae samples before sending them by CCAP to Coventry University to start the next stage, namely extraction.

2.3.3 Ultrasound-assisted Extraction (UAE)

The ultrasound-assisted extraction (UAE) technique was performed (Model XUBA1, UK). The bath was a rectangular container with annealed 50–60 kHz transducers at the bottom (34.29 cm x 10.16 cm x 30.5 cm). The samples were handled at room temperature. The ultrasonic wave led to a slight increase in temperature (i.e., between 40 and 50 °C). A round 1 L Erlenmeyer flask with a round bottom was filled with 5 mg of dried algae powder and 100 mL of 80% aqueous ethanol. This tube, which contained the mixture, was then submerged in the water in the tank of an ultrasonic device, which sonicated the mixture for 45 minutes at 42 KHz (135 W) and 25 °C using the ultrasonic bath. Next, the extracted substances were filtered (with Whatman No. 41 paper, 70 mm) via a cold Buchner funnel under vacuum. Then, 50 mL of 100% ethanol was used to rinse the filter cake, and the filtrate was transferred to a 1 L round-bottom flask containing 100 mL of 80% ethanol. The extraction procedure was then repeated for the residue. In a round-bottomed flask, the filtrates were mixed with an additional 50 mL of 80% aqueous ethanol, and the solvent was evaporated at 62 °C and 50 rpm for 45 minutes using rotary evaporation (BUCHI R-114, Germany). The 1.9 mg of extract was then held at 80 °C for 8 hours, moved to -20 °C until the TPC analysis, and used to treat cells.

Figure 2

Isolation of PH-rich extract (polyphenol) from brown algae by ultrasonic sound and 80% ethanol as solvent



Extraction of a PH-rich extract from *A. esculenta* and *S. latissima* by filling a round bottom with 100 mL of aqueous ethanol containing 80% ethanol and 5 mg of dried algae powder. The flask was then placed in an ultrasonic bath (45 minutes, 42 kHz (135 W), 25 °C) with water. Following sonication, the extract was filtered using a Buchner funnel and 70 mm Whatman filter paper under vacuum. Finally, the solvent was evaporated using rotary evaporation to produce powdered crude extract.

2.3.4 Preparation of Purified Polyphenol Extracts from Crude Extracts

To begin the purification process, 500 mg of crude extract were dissolved in 10 mL of ethanol. Afterwards, two volumes (20 mL) of chloromethane were added, and the mixture was shaken with a magnetic strip bar for five minutes. The mixture was then put into the separating funnel for one hour to obtain the purified fraction, after which it was separated into two layers. The dichloromethane layer was discarded, and the aqueous layer was collected in a clean beaker. Thereafter, two volumes of ethyl acetate were added to the aqueous fraction, followed by the addition of the magnetic strip bar and 5 minutes of shaking at room temperature. Then, the resulting mixture was filtered (with Whatman No. 41 paper, 70 mm) via a cold Buchner funnel using vacuum suction and Whatman No. 41 paper. The filtrates were collected in a round-

bottomed flask, and ethyl acetate was evaporated at 62 °C and 50 rpm for 30 minutes using rotary evaporation (BUCHI R-114, Germany). The 90 mg of extract was then preserved at 80 °C for 8 hours, moved to -20 °C until TPC analysis, and utilised to treat cells.

Figure 3: *Brown algae samples: A. esculenta and S. latissimia*

2.3.5 Determination of Total Phenolic Content (TPC) in Brown Algae

In the present study, the total phenolic content (Phlorotannin-rich extract) of brown algae was measured using the Folin-Ciocalteu Colorimetric method with gallic acid as references, according to the methodology described by Hoerudin (2004) with some modifications. The TPC was expressed as mg of gallic acid equivalents (GAE) per gram of sample extract.

2.3.5.1 Preparation of Standards. To determine the total phenolic content, expressed as gallic acid equivalent (GAE), a reference curve of known gallic acid values was created. The eight varied concentrations of the standard solutions were 0, 25, 50, 100, 150, 200, 250, and 300 mg/L. Initially, a stock solution of gallic acid was prepared by dissolving 500 mg of gallic acid in 10 mL of 95% ethanol. Secondly, to prepare the standard working solutions, 0, 0.1, 1, 2, 3, 4, 5, and 10 mL aliquots of a stock gallic acid solution (5000 mg/L) were pipetted sequentially into a 10 mL volumetric flask and diluted to volume with 95 percent ethanol. In order to produce the standard curve, 1 mL of each standard solution was pipetted into its own test tube. Thereafter, 200 uL of a 10% aqueous dilution of Folin-Ciocalteu reagents were added and well mixed for approximately 1 minute using a vortex mixer. After 3–8 minutes, 600 uL of a 250 mg/L anhydrous Na2CO3 solution was added, followed by 120 minutes of room-temperature incubation in the dark.

2.3.5.2 Preparation of Samples. 25 mg of each powdered crude or pure PH-rich extract of brown algae were diluted in 25 mL of 80% ethanol. Next, 100 uL was transferred from each ethanolic algae solution into a test tube and mixed for 5 minutes using an ultrasound device. Then, 200 uL of a 10% aqueous dilution of Folin-Ciocalteu reagent was added, thoroughly mixed with a vortex mixer for about 1 minute, and incubated at room temperature in the dark for 10 minutes. After 5 minutes, 600 uL of a 250 mg/mL solution of anhydrous Na_2CO_3 was added, followed by 120 minutes of dark incubation at room temperature. A blank sample was made by pipetting 900 uL of the extract's solvent, 95% ethanol, into a test tube. Following 120 minutes of incubation of the extract solution in the dark at room temperature, the absorbance of standards and extract solutions was measured at 765 nm against a zero absorbance blank using a UV/Visible spectrophotometer (Biochrom, USA). The following formula was used to calculate the final total of phenolic acid (PH-rich extract of brown algae):

$$\textbf{Total phenolic content (Mg GAE/g Algae)} = \frac{\boldsymbol{GAE\ (mg/L)\ x\ total\ volume\ of\ ethanol\ extract\ (mL)}}{\boldsymbol{Sample\ weight\ (g)}}$$

***2.3.5.3 Preparation of calibration standard solutions*.** The calculations were performed using a calibration curve using gallic acid equivalents (GAE), and the measurement of the concentration of the samples was carried out in triplicate. The mean results are reported in GAE using extract units of mg/L. However, calibration standard solutions were created by diluting the principle stock solution with distilled water to get a calibration standard solution of 25, 50, 100, 150, 200, 250, and 300 mg/mL of gallic acid solution. The absorbance was measured at λmax 765 nm against reagents to draw a standard plot (Table 1). The linear regression equation for the absorbance under the concentration was: y = 0.0049x + 0.2564, R^2 =0.9966.

Table 1

Linearity table of Gallic Acid Solution

Concentration (mg/L)	**Absorbance**
25	0.402
50	0.532
100	0.715
150	0.976
200	1.211
250	1.511
300	1.765

2.4 Materials and Methods

2.4.1 Examination of the Functional Responses of Human Skin Cells (NHDF) to S. latissima and A. esculenta Extracts

In this study, NHDF viability and proliferation in response to *S.latissimia and A.esculenta* brown algae extract (PH-rich extract) were evaluated extensively using the MTT assay.

2.4.2 General Cell Culture-Research on Wound Healing

Cell culture has been utilised to investigate cellular aspects of tissue repair, including proliferation and growth factor production (Kang et al., 2004), damage caused by an excess of toxins produced by inflammation (Thang et al., 2001), and the synthesis of wound healing biomolecules (Kim et al., 1997). These in vitro models allow the investigation of isolated components of the wound healing mechanism, a task that would be challenging to conduct under heterogeneous in vivo conditions. In addition, cell culture techniques can provide an initial evaluation of a material's capacity to promote wound healing (Howling et al., 2001). The medicinal agent dissolved in the growth medium is applied to monolayer cultures of skin cells. Prior to measuring a pharmacological response corresponding to wound healing, the cells are incubated for a predetermined time period. Most research has focused on dermal fibroblast, dermal endothelial, and epidermal keratinocyte cells as wound healing effectors to mimic wound healing *in vitro* (Gottrup et al., 2000).

2.5 Statistical Analysis

Statistical analyses were applied using GraphPad Prism 7 (GraphPad Software, USA). Two samples were examined and repeated three times in each technical experiment. Comparisons between multiple groups were performed using one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparisons test. The statistically significant level was set at $P<0.005$.

2.6 Materials

2.6.1 Normal Human Dermal Fibroblasts

NHDFs from adult donors were procured from PromoCell UK, Ltd., Woking, England (product code C-12302) and were cultured in sterile flasks (75 cm^2 surface) in fibroblast growth medium (C-23020, PromoCell) with supplements (C-39325, PromoCell) in accordance with the manufacturer's protocol. 3500-7000 cells/cm2 were utilised, and the cell culture was maintained at 37 °C and enriched with 5% CO2 of air atmosphere.

2.6.2 Cell Culture and Cell Handling Materials

Incubator

Cabinet was used to grow cell culture and maintain temperature for both the incubation of NHDF and the equilibration reagents.

Flow cabinet

A Microflow Class II laminar flow cabinet (class II, ESCO) was utilized for the preparation of cell culture media and reagents and subculturing cells.

Cell culture media and reagents

Fibroblast Basal Media (FBM), Fetal Bovine Serum (FBS), Trypsin, Trypsin Neutralising Solution (TNS), and Hanks Balanced Salt Solution (HBSS) were purchased from PromoCell UK Ltd., Woking, England. Hydrogen peroxide (5 M, 30% w/w), trypan blue (0.4%), and ferric sulfate (P) were purchased from Sigma-Aldrich.

2.6.3 Cell proliferation

MTT assay kit

MTT(3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium) is the proliferation kit used for spectrophotometric quantification of cell proliferation and cell viability. MTT assay kit were purchased from Sigma Aldrich.

Microplate reader

A microplate reader was used for the MTT assay to measure cell proliferation and viability, in which the amount of formazan (directly proportional to the number of viable cells) was determined by recording variation in absorbance at 570 nm.

2.7 Methodology

2.7.1 Thawing and plating of NHDF cells

Cryopreserved vials of NHDFs were taken from a storage tank of liquid nitrogen using eye and hand protection, and the vial cap was opened slightly to release any liquid nitrogen, and then retightened in the cabinet II. The lower half of the cell vial was then rotated in a 37 °C water immersion for two minutes. The cell vial was then transferred from the water immersion to Cabinet II, where its exterior was decontaminated with 70 percent alcohol in a sterile Biological Safety Cabinet. After this, the cell vial was resuspended five times using a 2 mL pipette, and the 1 mL of cell was pipetted from the vial into a T-75 flask containing 15 mL of Finroblast Growth Medium (116-555, Promocell). The T-75 flask was placed in a 37 ^{o}C, 5% CO_2 Humidified incubator, and the medium was changed after 24 hours or overnight to remove all traces of DMSO. The Fibroblast Growth Medium was changed every 2 days until the cells reached 80% confluency, to subculture the cells.

2.7.2 Subculturing of NHDF cells

Prior to use with the cells, the incubator was allowed to equilibrate all reagents, and all NHDFs were handled in a laminar II flow cabinet of class II. The DetachKit of PromoCell was placed 30 minutes at room temperature of the refrigerator. NHDFs were cultivated to a confluence of approximately 80%. The media

carefully aspirated culture flask and the cell were rinsed with 100 uL HBSS solution per cm^2 of vessel surface to wash the cells, and the vessel was carefully agitated for 15 seconds. Then, HBSS was aspirated carefully from the flask, and 100 uL Trypsin/EDTA per cm^2 was added, NHDFs were observed under a microscope for three minutes. After the majority of cells were detached from the flask by the Trypsin/EDTA solution, 100 ul of trypsin neutralising solution was added per cm2, and the resulting cell suspension was transferred to a 15-mL centrifuge tube at 220*g for 3 minutes. After that, the supernatant was discarded, PromoCell Growth Medium was added, and the cells were resuspended. At the end, the cell number was determined using a haemocytometer with regard to the suggested seeding density in new cell vessels containing 37 °C-warmed PromoCell Cell Growth Medium. The vessels were kept in an incubator (37 °C, 5% CO2) until they were utilised. In all experiments, cells between the third and fifth passages were used.

2.7.3 Counting cells with Haemoxytometer

2.7.3.1 Background. A haemocytometer is a tool used for manual cell counting. It is composed of a glass slide with a small square chamber etched into it. The volume of the square is 0.1 mm (10–4 mL). The chamber's floor is divided into nine sections, with the cell type dictating which sections are used for counting.

2.7.3.2 NHDFs Counting. 10 mL of cell suspension was combined with an equal volume of Trypan Blue (0.4%), and 10 mL of the resulting solution was pipetted into haemocytometer. When counting NHDFs, only the four exterior squares were used. The cell number per mL (C ml) was calculated using the mean cell number (Cav), as shown in the following equation:

$$C\ ml = 2\ Cav \times 10\text{^}4$$

A factor of 10^4 was required to convert the volume (counted 10^4) to 1.0 ml, and a factor of 2 was added to account for the Trypan blue dilution.

2.7.4 Preparation of MTT Solution

By dissolving 5 mg of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in 1 mL of PBS, a 12 mm stock solution was created. This stock solution was sufficient for 100 reactions (each requiring 10 mL). Once prepared, the MTT solution should be stored at 4 °C and away from light for four weeks at 4 °C.

2.7.5 Preparation of samples for treatment

2.7.5.1 MTT assay. Cell proliferation of NHDFs after treatment with different concentrations of PH-rich extract from brown algae was measured using MTT assay. The 96-well MTT assay is a modification of the method proposed by Burres and Clement (1989). Prepare a 12 mM solution by dissolving 5 mg of MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] in 1 mL of PBS. All samples were filtered using a 0.2-um syringe filter. Seed a total of 5 x 103 NHDF cells into a 96-well plate in a total of 200 uL of NHDF medium and incubate for 24 hours to grow as a monolayer. After incubation of the cells in suspension for 2–3 days' incubation (37 °C, 5% CO_2, 95% air) to reach full confluency, the cells were then treated with various concentrations (25, 50, 100, and 200 mg/uL) of a crude and pure PH-rich extract of two species of brown algae (*S. latissima* and *A. esculenta*). Then, after 24 hours, the MTT assay was performed by discarding the 200 uL media and replacing it with 100 uL of fresh media for each well, and adding 10 uL of MTT test solution to each well of the 96-well plate to obtain a total reaction volume of 110 µL. The plate was then returned to a tissue culture incubator for 8 to 24 hours (37 °C, 5% CO2, 95% air) to allow viable cells in the culture to enzymatically reduce the tetrazolium salt. Then 100uL/well of the MTT solubilizer Dimethylsulfoxide (DMSO) was added and gently mixed (Dwivedi et al., 2015). The blue formazan precipitate was then dissolved overnight in an incubator (37°C, 5% CO2, 95% air). The absorbance of each well was then measured using a VERSA MAX microplate reader (165 qt, 570 nm; Multiskn RE, USA). The absorbance indicates the viability of NHDFs cells because it converts yellow MTT to purple formazan. The insoluble purple formazan was dissolved in DMSO to produce a pink-to-purple-coloured solution, which was quantified by measuring its UV light absorption. The percentage of viable cells was calculated according to the following formula:

$$\% \text{ Cell viability} = \frac{\text{Absorbance of treated cells}}{\text{Absorbance of the untreated cells}} \times 100$$

Results

3.1 Materials Extraction Yields

Table 2 provides the material weights used and obtained during extraction procedures: Quantities used/obtained during the drying of *A. esculenta and S. latissimi* brown algae material

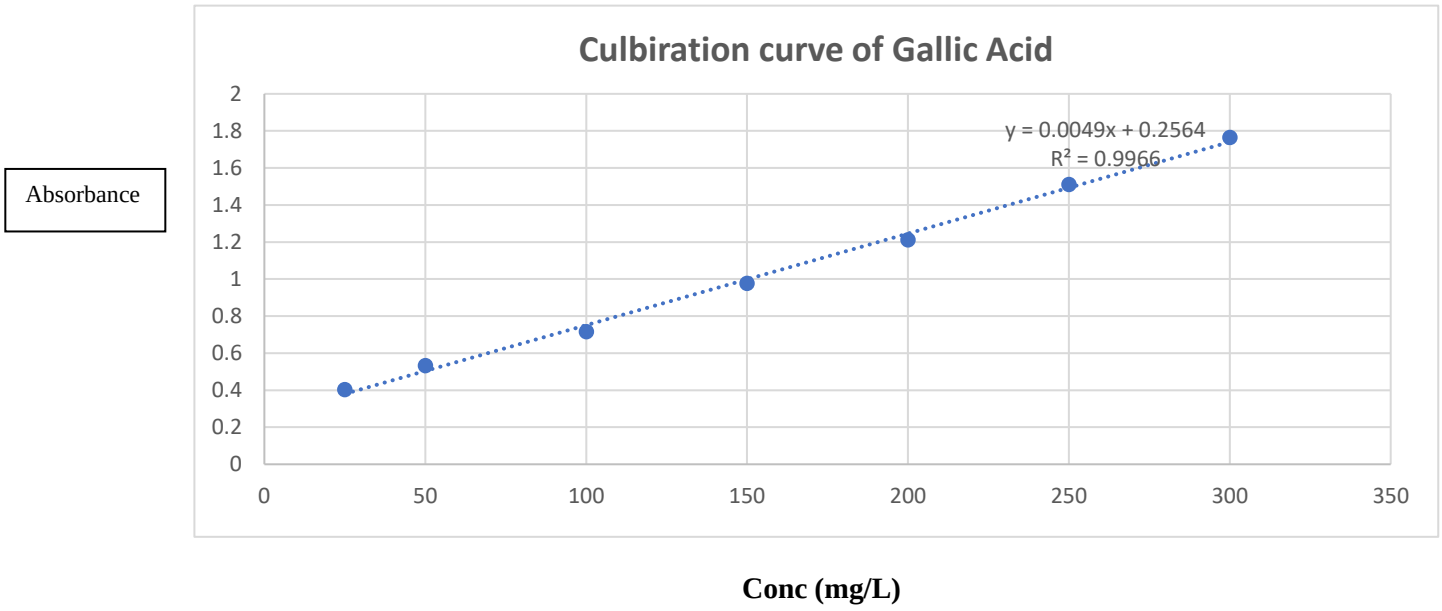
Table 2

Weight of Materials obtained after drying, extraction, purification of algal Material

Algal (PE) (mg)	weight after drying (g)	weight after grinding (g)	weight of material for extraction (g)	weight of extracted material (g) Crude extract (CE)	Weight of material for purification (mg)	Weight of pure material
S. latissimi	**6.2**	**3.2**	**2.8**	**1.96**	**500**	**90**
A. esculenta	**5.3**	**5.2**	**2.8**	**0.9**	**500**	**50**

Figure 4

Linearity Curve of Gallic Acid Solution



3.2 Total Phenolic Content of the Extract (Phlorotannin-Rich Extract)

The total phenolic content was determined using the Folin-Ciocalteu method and represented as milligrams per gram of dry weight (dry seaweeds) based on the standard curve of gallic acid, which was estimated to be milligrams per gram of GAE. In order to evaluate the total amount of phlorotannin-rich extract from S. latissimi and A. esculenta crude and pure extracts, the following calibration curve of gallic acid equivalents (GAE) was developed using the standard curve equation y = 0.0049x + 0.2564, R^2 =0.9966 (Figure 3). As shown in Tables 2 and 3, the means of total phenolic acids (PH-rich extract) of S. latissimi and A. esculenta varied between 24.2 and 40.2 mg GAE/g. The lowest quantity found was 24.2 mg GAE/g for the crude extract of S. latissimi, while the maximum level was 40.2 mg GAE/g for the pure extract of A. esculenta. Additionally, while there was also some variation between the crude and pure extracts in both species, the S. latissimi showed a greater amount of pure PH-rich extract, which contained 13.4 mg GAE/g, compared to its crude extract, which contained 10.3 mg GAE/g, whereas the A. esculenta presented a much higher amount of pure PH-rich extract, which contained 40.2 mg GAE/g, compared to its crude extract, which contained 37.2 mg GAE/g.

Table 3

Total Phenolic Content

Algae	**Sample Absorbance**	**Calculated Conc. (mg/L)**	**Mean**	**SD**
Crude extract	**0.307**	**10.3**	**10.3**	**2.2**
S. latissimi	**0.294**	**7.7**		
	0.320	**13**		
Pure extract	**0.374**	**24**	**13.4**	**7.6**
S. latissimi	**0.302**	**9.3**		
	0.290	**6.9**		
Crude extract	**0.465**	**42.6**	**37.2**	**4.4**
A. esculenta	**0.432**	**37.3**		
	0.412	**31.8**		
Pure extract	**0.487**	**47.1**	**40.2**	**6.6**
A. esculenta	**0.423**	**34**		
	0.451	**39.7**		

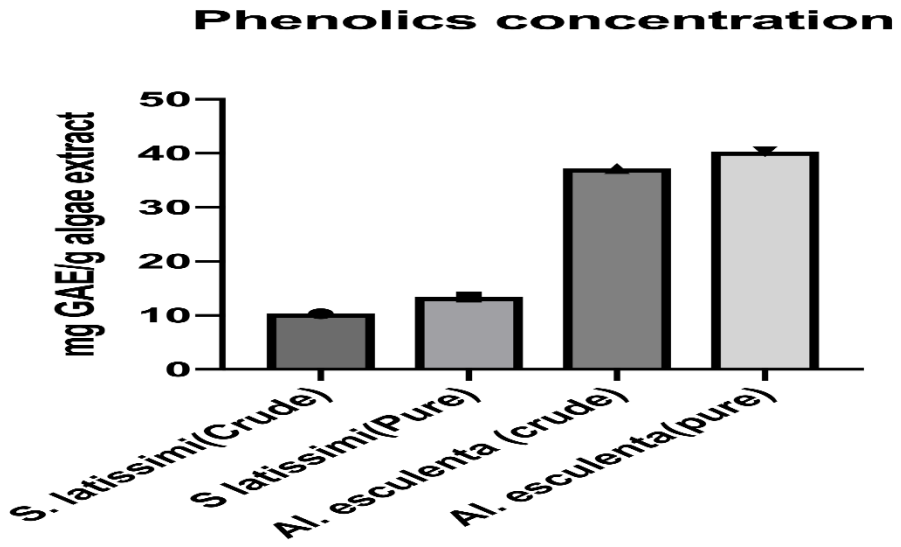
Table 4

Mean Concentration of Phenolic (Ph-rich extract)in each algae extract

Crude extract and pure extract	**mg GAE/ g algae extract**
Crude extract *S. latissimi*	10.3 ± 2.2
Pure extract *S. latissimi*	13.4 ± 7.6
Crude extract *A. esculenta*	37.2 ± 4.4
Pure extract *A. esculenta*	40.2 ± 6.6

Figure 5

Phenolic Content (PH-rich extract), expressed as mg GAE/g of Extract.



3.3 NHDF Cell Viability

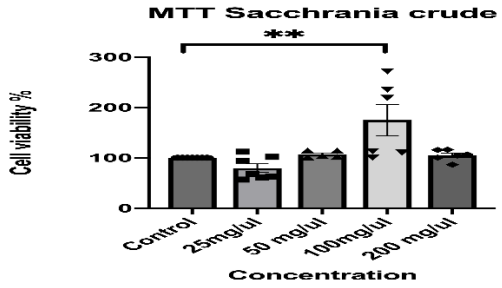
3.3.1 Results of the MTT Assay

In this investigation, four brown algae extracts (crude and pure), derived from *S. latissima* and *A. esculenta*, were evaluated on NHDF cells and compared to the control (Figure). (A-D).

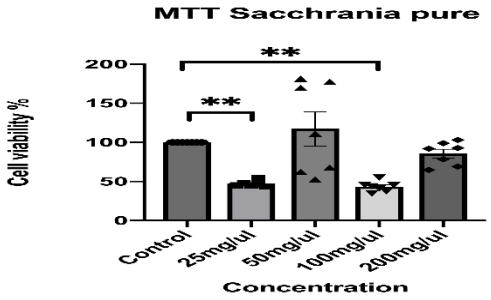
Figure 6

Relative percentage of NHDF viability treated with Saccharina (Saccharina latissimi) and Alaria (Alaria esculenta) extract (PH-rich extract):

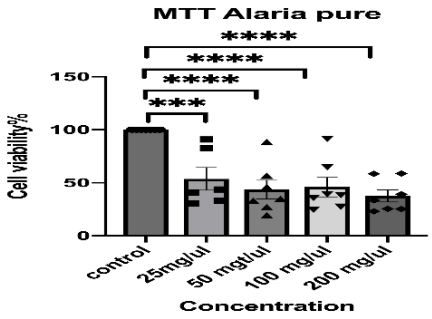
(A) Relative Cell viability of NHDF cells with crude Saccharina extract



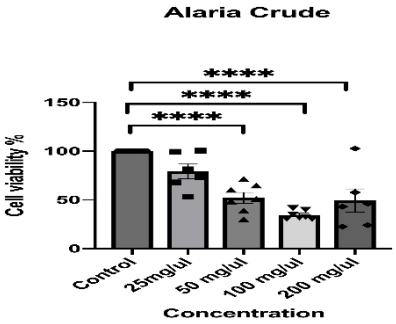
(B) Relative Cell viability of NHDF cells treated treated with Pure Saccharina extract



(C) **Relative Cell viability of NHDF cells treated with Crude Alaria extract**



(D) Relative Cell viability of NHDF cells treated with Pure Alaria extract



(A) *Saccharina* crude, (B) *Saccharina* pure, (C) *Alaria* crude, (D) *Alaria* pure of (PH-rich extract) At different concentrations (25 mg/ul, 50 mg/uL, 100 mg/uL, and 200 mg/uL), the average percentage related viability (±SEM) of seven replicates (n=7). The percentage of cell viability stimulated by *Saccharina* (A) crude extract 100 mg/uL was significantly higher than the positive control, and pure extracts (B) at 25 mg/mL and 100 mg/mL of *Saccharina* showed that NHDF decreased viability rate compared to the control. In contrast, all other extracts of *Alaria*, both crude and pure (PH-rich extract), induced lower percentages of cell viability than the positive control, except the crude extract of *Alaria* at 25 mg/mL, which showed no difference from the control group. The positive control represents untreated cells cultured in low serum media (RSM). Significantly level ****P<0.0001, ***P<0.001 and *P<0.05 compared to positive control. MTT: [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide], *Saccharina*: *Saccharina latissima*, *Alaria*: *Alaria esculenta* , PH-rich extract: polyphenol extract rich in phlorotannin, RSM: reduced serum medium.

To investigate cell viability and proliferation and select the optimal extract concentration for the cell culture experiments, this study examined the effects of a PH-rich extract of *S. latissima* and *A. esculenta* on cell viability after incubation for 24 hours by -(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (MTT) assay. As seen in (Fig. 5A) S. latissima crude extracts (PH-rich extract) enhanced cell viability compared to the control, which is a culture of untreated cells. The crude extract (PH-rich extract) of S. latissima at 100 mg/uL ($P < 0.0009$) showed higher cell viability than the control compared to the control (Fig. 5A). In addition, it is noticeable that Figure 5 (C), which presents the normal human dermal fibroblast decreased viability rate at 25 mg/mL and 100 mg/mL compared to the control. On the other hand, regardless of concentrations, NHDF cells treated with crude and pure extracts of *A. esculenta* were less viable than control cells (Figs. 5C and 5D). All A. esculenta-derived treatments significantly ($P<0.0001$) decreased NHDF viability compared to the control group, except for the crude extract of *A. esculenta* at 25 mg/mL, which showed no difference from the control group.

Discussion

Skin is the largest organ in the body and creates a barrier that separates our interior body from the external environment. It protects us from cold, sun, moisture, weather, and physical (stress), chemical, and biological (necrotic state, pathogenic bacteria existence, hypoxic environment) factors if entering the body may be harmful to our body (Intayoung et al., 2016). However, if there is any break (also known as a wound) on the surface of the skin, the measure of protection may not be sufficient (Intayoung et al., 2016). In clinical practice, wounds are generally perceived as a significant problem. While standard wounds recover within a few days, chronic wounds are significant economic and social challenges. Chronic wounds are a significant burden for patients and the NHS in the United Kingdom. Approximately 200,000 patients in the United Kingdom have a chronic wound. It is well-documented that their quality of life is affected (Franks and Morgan, 2003). Therefore, it is essential to discover novel natural products that are more effective and less expensive (Addis et al., 2020, Alfarrayeh et al., 2022).

According to this point, diabetic people, delayed wound healing or non-healing wounds represent one of the most significant problems that reduce quality of life and raise healthcare expenses. In addition, diabetes can impede the healing process or increase the risk of infection in the affected area, leading to prolonged hospitalisation (Jaul et al., 2018). According to previous research, diabetic wounds demonstrate delayed wound healing, reduced collagen levels, and altered oxidative processes (Blakytny & Jude, 2006; Dindar et al., 2017). Hence, the chemical basis of healing promotion is the topic of ongoing research. Previous studies suggested that, the minerals, vegetable proteins, vitamins, fatty acids, sugar, volatile compounds, and phenolic compounds found in microalgae serve as antioxidants, antibacterials, antiviral agents, skin regenerators, immune modulatory agents, immunostimulants, and scavenge destructive free radicals responsible for cell death (ABD El-Baky et al., 2009; Hirahashi et al., 2002; Kim et al., 1998; Li et al., 2005; Plaza et al., 2009; Qishen et al., 1998; Reddy et al., 2003; Singh et al., 2005; Subhashini et al., 2004). In particular, brown macroalgae or seaweeds are distinguished by their rapid growth and large concentration of minerals, carbohydrates, and polyphenols, especially phlorotannin, which are significant components for use in feed, food, and medicine (Penalver et al., 2020). Additionally, the most previous treatments for treating the wound may take longer, or the infection may spread. Therefore, this study advances the use of medicinal algae extract to enhance the development of novel therapeutic agents. It is suggested that novel phlorotannin extracts from the brown algae S. latissima and A. esculenta represent a promising approach for wound healing, particularly in diabetic patients who suffer from delayed wound healing. This is the first study to examine the potential wound healing activity of a PH-rich (polyphenol) extract of S. latissima and A. esculenta in terms of related cell viability and the proliferation of human skin cells (NHDFs).

The main findings of this study were as follows. In the first part of the investigation, *S. latissima* and *A. esculenta* were characterised by phytochemical compositions and total polyphenol content (PH-rich extract). The crude PH-rich *S. latissimi* extract contained 10.3 ± 2.2 mg GAE/g, and the pure PH-rich extract contained 13.4 ± 7.6 mg GAE/g. On the other hand, the crude PH-rich *A. esculenta* extract contained 37 ± 4.4 mg GAE/g and the pure PH-rich extract contained 40.2 ± 6.6 mg GAE/g. Secondly, the MTT application confirmed that PH-rich extracts from *S. latissimi*, especially the crude extract at 100 ug/mL, have significant potential to improve NHDF proliferation and viability, which are important attributes of a potential therapeutic for the treatment of chronic wounds, specifically in diabetic patients. Moreover, this study suggested that PH-rich extracts from *A. esculenta* may be considered as candidate biomaterials as regulator factors for cell viability and proliferation.

Proliferation and migration of cells are essential to the healing process. Fibroblasts are essential for the deposition of collagen, which is necessary for tissue regeneration after injury because collagen is essential for restoring the anatomy and function of wounded tissues (Suvik et al., 2012). In the early phase of proliferation, fibroblasts engage in cell division and migration. On the third day following an injury, a large number of fibroblasts begin to proliferate in the injury tissue and generate a large quantity of collagen, which determines the wound healing activity (Panwar et al., 2013). Our results showed that the PH-rich extract strongly stimulated cell viability and proliferation.

4.1 Total Phenolic (PH-Rich Extract) Content of Extracts

Total phenol content (TPC) varies significantly amongst various species of seaweed, which contain between 0.4 and 24.2 g PGE per 100 g of extant material (Wang et al., 2009). In general, brown algae have a higher amount of polyphenol than green algae and red algae (Wang et al., 2009). Researchers suggested that the species of seaweeds categorised into two groups based on their total phenolic content: low-phenolic species (2% dw) and high-phenolic species (>2% dw) (Roleda et al., 2019). *A. esculenta* has generally exhibited a greater quantity of extracted polyphenols for all extraction solvents and drying processes utilised in previous investigations (Roleda et al., 2019). In this study, the A. esculenta freeze-dried sample, which was extracted in 80% ethanol, contained a significantly higher amount of PH-rich extract than the *S.latissima* freeze-dried sample.

This finding is similar to the result of the TBT4500 project carried out in the fall of 2019 that presented the phenolic compound content of freeze-dried *A.esculenta* in 80% acetone (36.86 ± 2.69 mg PGE/g dry algae) was substantially higher than that of *S.lattissima* in 80% acetone (36.86 ± mg PGE/g dry algae), the values represent the average sample collected in spring, summer, and autumn(Roleda et al., 2019). In this study, the phenolic contents of PH-rich extracts of freeze-dried *S. latissima* and *A. esculenta* extracted in 80 % ethanol were found to be 10.3 2.2 mg GAE/g dried algae and 37.2 4.4 mg GAE/g dried algae, respectively. This is equivalent to the literature values (Roleda et al., 2019) and is also confirmed by Stévant et al. (2017), who stated that *A. esculenta* has five times more polyphenols than *S. latissima* (Stévant et al., 2017).

In this study, 80% ethanol was used as the extraction solvent, since it has been reported that the extraction solvent used affects polyphenol yield (Wang et al., 2009). As phenols dissolve better in less polar solvents than water, organic solvents such as ethanol and acetone are suggested for the extraction of polyphenols (Wang et al., 2009). Additionally, it has been reported that acetone and ethanol improve polyphenol output by preventing the formation of polyphenol-protein complexes or by disrupting hydrogen bonds (Hagerman, 1988). In the present study, 80% ethanol extracts yielded the highest levels of phenolic compounds compared to prior studies that utilised water as the extraction solvent, resulting in the extraction of water-soluble components such as proteins, polysaccharides, and organic acids (Chirinos et al., 2007).

Ethanol, a highly polar solvent, was used to extract a PH-rich extract of brown algae by targeting phenolic chemicals, one of the primary categories of secondary metabolites in brown algae. Water enhances the activity of polyphenol oxidase, an enzyme that hydrolyzes polyphenols (Derardja et al., 2022): Therefore, only low quantities of water are necessary to form the solvent. Alcohol was used to preserve the phenolic compounds deemed to be the active chemicals in this investigation since it has no effect on enzyme activity.

Because methanol is more polar than ethanol, it can be used, but its cytotoxicity makes it unsuitable. Hence, ethanol was employed rather than methanol. In the meantime, the minor increase in hydrophobicity enhances the breakdown of cells and the penetration of the solvent through the extracted cell membrane (Apak et al., 2007).

4.2 MTT Assay and Cell Viability and Proliferation

This study used the MTT assay to test the wound-healing ability of PH-rich extracts *in vitro*. The MTT assay served various purposes, including measuring the efficiency of PH-rich extracts of two species of brown algae (*S. latissimi* and *A. esculenta*) on NHDF cells' viability and proliferation, as well as identifying concentration ranges for subsequent experiments.

The MTT assay is a novel technique for measuring the metabolic activity of cells by estimating the rate of reduction of tetrazolium into formazan through mitochondrial enzyme activity. This is only feasible in cells with functioning respiration (Muniandy et al., 2018). MTT reagents are lysed through the activity of metabolic cells to produce formazan quantities that are proportional to the number of cells in a homogeneous cell population over a wide range (Mosmann, 1983). As active cells create more formazan than resting cells, this measurement matches cellular activity more precisely. It is therefore a reliable approach for assessing cell viability or possible cytotoxic effects (Mosmann, 1983).

The overall findings of improved cell viability indicated that 100 mg/uL crude *S. latissimi* extract (PH-rich extarct) showed the highest cell viability compared to the control (untreated cells) (Figures 5A), suggesting that the cells became very active and motile, indicating that their metabolic activity was extremely high and was a direct result of treatment. The high metabolic rate of the NHDF cells appeared to trigger the increased absorbance readings, as mitochondrial activity is directly proportional to the oxidation of the MTT dye and thus to the colour intensity. This finding is supported by previous research, which indicated that polyphenols found in brown algae enhance cell proliferation and improve cellular activities (Kang et al., 2012; Yeo et al., 2012). Particularly, polyphenols have been shown to significantly stimulate the expression of the growth factor insulin-like growth factor-1 (IGF-1), which promotes cell growth, proliferation, and differentiation (Bak et al., 2013; Shin et al., 2016). In addition, as seen in Figure 5 (C), which presents the normal human dermal fibroblast's decreased viability rate at 25 mg/mL and 100 mg/mL of PH-rich extract of *S. latissima* pure extract compared to the control. This indicates that some compounds of polyphenol in PH-rich extract, especially phlorotannin, may have been affected after purification, resulting in a change in the effects on cell viability.

In contrast, as shown in Figures 5C and 5D, inhibition of cell proliferation compared to the control was seen in NHDF cells treated with a PH-rich extract of both pure and crude of *A. esculenta* ranging from 25

ug/ml to 200 ug/ml except the crude extract of *Alaria* at 25 mg/mL, which showed no difference from the control group. This result indicates that the PH-rich extract from *A. esculenta* may be a regulatory factor for several diseases. Using a similar approach, Lee et al. (2019) found that phlorotannin shows potential as an anti-fibrotic agent through regulation of cell proliferation and protein expression levels of fibrosis phenotype markers (collagen type I and α-SMA) via blocking of SMAD 2/3 in TGF-β-stimulated Hs680.Tr human tracheal fibroblasts. Therefore, PH-rich extracts from *A. esculenta* may be considered candidate biomaterials for several diseases as regulated fibrotic markers (Lee et al., 2019).

According to the author's knowledge, this is the first project to effectively assess the effects of *S. latissimi* extracts on NHDF in terms of cell viability, proliferation, and potential for wound healing. These findings demonstrate that the PH-rich extract of *S. latissimi* presents a promising approach for a wound healing environment around the wound site by increasing cell proliferation. In addition, the 100 mg/uL concentrations of crude and pure extracts respectively are suitable concentrations to increase the cell proliferation of NHDF. This promising finding of this study is the first stage of the development of the idea of wound healing plasters for diabetic patients. In addition, this investigation suggested A. esculenta may be a regulatory factor for several diseases because it has a high amount of PH-rich extract and the ability to decrease cell viability and proliferation.

4.3 Conclusion

In the previous research, PH-rich extracts have been investigated and demonstrated to have critical biological effects, including antioxidant, anti-inflammatory, and antibacterial properties. Nevertheless, neither the impact of PH-rich extracts on NHDF growth nor their application to wounds have been investigated previously. Hence, the present study investigated the effect of *S. latissima* and *A. esculenta* (brown algae) on cell proliferation of NHDF. Our findings demonstrate, the wound healing potential of a PH-rich extract of S. latissimi on NHDF using an in vitro MTT assay. The proliferation of NHDF was slightly increased when treated with crude PH-rich extract concentrated at 100 mg/ul, compared to the untreated positive control. On the other hand, the results show that cell proliferation decreases when the amount of PH-rich extract from Alaria esculenta is increased compared with the control. Thus, the findings suggest that these PH-rich extracts may be promising candidates for use in wound dressings, particularly in diabetic patients who suffer from delayed wound healing. In addition, the results show that the crude PH-rich *S. latissimi* extract contained 10.3 ± 2.2 mg GAE/g and the pure PH-rich extract contained 13.4 ± 7.6 mg GAE/g. In contrast, the crude PH-rich *A. esculenta* extract contained 37 ± 4.4 mg GAE/g and the pure PH-rich extract contained 40.2 ± 6.6 mg GAE/g. However, the comprehensive results suggest that the PH-rich extracts from *Sachrania latissima* may be good candidates for use in wound dressings. Moreover, our study suggested that the PH-rich extract from *Alaria esculenta* can be used as a regulatory factor for several diseases. Further research should examine in vitro the influence of PH-rich extracts on wound healing using a scratch assay, an investigation of NHDF cell proliferation in diabetic patients, and in vivo application in the mouse before the extracts are applied as a wound healing plaster for diabetic patients.

4.4 Limitations and future work

In the future, intense research is needed to examine in vitro the influence of PH-rich extracts on wound healing using a scratch assay to mimic wound healing in vitro, an investigation of NHDF cell proliferation in diabetic patients, and in vivo application in the mouse before the PH-rich extract is applied as a wound healing plaster for diabetic patients. The limitations of this study mainly include contaminated NHDF cell culture and growth medium and insufficient time and funds to get new media, which resulted in not performing the scratch assay, which is an essential experiment in this investigation In addition, there is no device for high-performance liquid chromatography (HPLC) that is used for measuring the concentration of individual polyphenols such as phlorotannin. Therefore, this study just measured the total polyphenol as a phlorotannin-rich extract, as suggested in a previous research paper that found a brown algae to have a good amount of phlorotannin in the total polyphenol of *S. latissima* and *A. esculenta*. In future studies, it would be interesting to measure the amount of phlorotannin individually in *S. latissima* and *A. esculenta.*

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