

Investigating Plant-based Medicines for Wound Healing with the Use of Cell Culture Technologies and *In vitro* Models: A Review

T T Phan,^{*MD}, S T Lee,^{**FAMS., MBBS (Adel) FRCS}, S Y Chan,^{***PhD}, M A Hughes,^{†PhD}, G W Cherry,^{‡DPhil}

Abstract

Introduction: Cell culture and molecular technologies are basic yet sophisticated research tool used to investigate plant-based medicine for wound healing. **Methods:** Cell viability and proliferation assay is used to determine whether there are any positive effects and to discover what is the limiting cytotoxic concentration in vitro. The scratch technique, fibroblast-populated collagen lattices and aortic rings embedded gels are used as the in-vitro models of wound re-epithelialization, contraction and angiogenesis. The immunofluorescence, immunoblotting and organotypic culture can be used to detect expression of specific proteins that are modulated by plant extracts during the wound healing process. **Main Findings:** Given the dynamics of the wound healing process, cell culture and molecular technologies are advantageous in providing us with detailed studies and analysis of each intricate process. **Conclusion:** The scientific approaches for the study of traditional plant-based remedies for wound healing will provide us an important platform for rigorous testing and evaluation of their clinical efficacy based on accepted rules of evidence.

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Introduction

Burn trauma, accident wounds and chronic wounds remain major problems in many countries. In the United States, approximately 2.5 million people with burn injury seek medical care with 80,000 hospitalisations and 12,000 deaths annually. The National Burns Centre in Singapore General Hospital, Singapore admits on average 300 patients a year, and about 25% of whom have sustained major burns. Patients with large surface area wounds continue to provide significant challenges in both applying short-term wound closure and preventing long-term wound contraction and scar formation. Foot ulceration and amputations are important causes of morbidity in diabetic patients, up to 10% have suffered foot ulceration. Diabetes accounts for up to 50% of non-traumatic leg amputations and 1% of diabetic patients have undergone amputation, which is about 15 times more than the rate in the general population.¹ In order to decrease the mortality rate in burn and to improve the quality of life of patients with problematic

wounds, one of the most important goals is to enhance the wound healing and the regeneration process. Over the last two decades many advances have been made in the understanding of molecular and cellular biology as well as biochemistry, physiology and pathology of wound healing. In recent years, application of advanced technologies (tissue engineering of skin, recombinant growth factors, gene therapy, etc) in wound care has been developing. Survival rate and improvement in quality of life of many patients with large burn injury and non-healing chronic wounds have increased in the United States and Western Europe. However, wide clinical use of these technologies is still limited and not practical due to costs. Peacock² pointed out that there was a “glaring gap” between basic research and clinical application in the biology of wound healing. For burn treatment alone, US\$5 billion are spent every year worldwide.³ In the United Kingdom, the costs of managing chronic leg ulcers alone are estimated at £236 million per annum.⁴ Foot lesions are now the commonest cause of

* Research Scientist

** Associate Professor and Senior Consultant
Department of Plastic Surgery/National Burns Centre
Singapore General Hospital

*** Assistant Professor
Department of Pharmacy, National University of Singapore

† Clinical Scientist

‡ Chairman of Wound Healing Programme
Wound Healing Institute

Department of Dermatology, The Churchill, Oxford, United Kingdom

Address for Reprints: Dr Phan Toan Thang, Department of Plastic Surgery, Singapore General Hospital, 1 Hospital Drive, Singapore 169608.

E-mail: gplsgj@sgh.gov.sg

hospital admissions amongst diabetic patients in the West and represent a major component of the diabetic health care expenditure, estimated at over US\$500 million and £15 million per year in the USA and the UK, respectively.¹

Wound care can be traced back to early civilisations. In China, India, Vietnam and Europe there is written history of wound management dating back many centuries.⁵⁻⁹ Most of these treatments were based on the use of herbal remedies. Today, traditional health systems are considered to be “one of the surest means to achieve total health care coverage of the world population, using acceptable, safe and economically feasible methods”—World Health Organization, 1978 and “80% of the world’s population depend on traditional medicine for its primary health care.”—World Health Organization, 1993.

Research programmes and application of wound healing agents prepared from medicinal plants have been carried out in many developed and developing countries in order to seek new avenues for improving wound healing and at the same time reducing the financial burden of health care. For example, the Wound Healing Institute in Oxford, UK has cooperative studies with units in China and Vietnam to screen traditional remedies.¹⁰ The beneficial effects from *Aloe Vera* were published by a research group in the Shriners Burns Institute in Galveston, Texas, USA.¹¹ Enhanced wound re-epithelialisation and neo-vascularisation of the extracts from Traditional Chinese Medicine mole crickets—commonly used to treat non-healing wounds was investigated in the University of Saarland, Germany and Louisville, KY, USA.¹² The enhancement of vascularisation by the European marigold extract was studied in the ConvaTec Wound Healing Research Institute in the UK.¹³ The Burns Unit in the Red Cross Hospital, Beverwijk, the Netherlands is also interested in Chinese herbs.³ The clinical and basic trials of moist exposed burn ointment (MEBO), a Chinese herbal ointment for treatment of burns and ulcers, have been carried out in the National Burns Centre, Singapore General Hospital, Singapore and Jerusalem, Israel.^{14,15}

Plant-based medicines for wound healing have been practiced for hundreds of years, but they need scientific evaluation for the interpretation and elucidation of their clinical efficacy. In recent years, advances in cell culture and molecular technology have revolutionised biological and pharmacological research in wound healing. A healing wound is a very complex biological system and requires both *in vivo* and *in vitro* models. The cell culture and *in vitro* models are systems, which allow more detailed studies on single elements in the repair process, and can simplify complex process.¹⁶

In this paper, we illustrate how plant-based medicines used to enhance wound healing can be examined in a

scientific manner using cell culture technology and *in vitro* wound healing models.

Methodology

Preparation of Plant Extract Material for In vitro Experimental Study

This step is crucial and indispensable in cell culture study. Traditional remedies for wound healing are manufactured under various formulations. They are extracts from a single part of a single plant (leaves, root, stem or bark) or mixtures of different plant extracts. These extracts are manufactured into the following forms: aqueous, alcohol extracts, powder, suspensions or ointments. For ease in *in vitro* cell culture study, plant-based drugs should be soluble in culture medium or water and sterilised (filtration). Some drugs under ointment formulation could be solubilised in detergent at high temperature. Essentially, the detergent should be included as control in experiments as some active components of the plant-based drugs may be deteriorated.

Endotoxin Assay

Endotoxin contamination can occur during the preparation and formulation of plant extracts. Endotoxin is claimed to have effects on *in vitro* cell culture. During experiments it is necessary to run endotoxin test to ensure that the observed positive effects on cell culture are due to the extract itself and not to endotoxin contamination.

Assays for Cell Viability and Proliferation

When several concentrations of a potential stimulatory or inhibitory agent are being investigated, cell counting in a haemocytometer is very time consuming. Therefore, various indirect methods of determining cell growth and viability, such as uptake of tritiated thymidine, determination of DNA or protein content or various colorimetric assays are commonly used.^{17,18} We generally use the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide metabolic assay or MTT assay to determine cell growth and to assess cell damage as described by Hansen et al.¹⁹ Using this method, the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide is reduced by microsomal enzymes of living cells that require reduced pyridine nucleotides. Succinate can also act as an electron donor in 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide reduction through mitochondrial succinate dehydrogenase.²⁰ With this mechanism, the highly coloured end products called blue formazans are formed and eluted from the cells and quantified in a spectrophotometer. A large number of samples can be rapidly and simply analysed. When using the MTT assay, caution needs to be exercised because several catechols and the flavonoid quercetin were found to reduce the MTT in serum supplemented medium

that resulted in fake readings on the spectrophotometer.²¹ We also encountered the same problem in testing antioxidant effect of extract from *Buddleja globosa* on fibroblasts using the MTT assay.²² In such cases, an alternative colometric method, such as the neutral red uptake assay, could be employed.

In cell proliferation studies, cell counts with trypan blue dye and haemocytometer should be performed in parallel with MTT assay for the most stimulatory dose of extract to confirm the correlation between cell number and MTT response.

Study of Effects of Plant-based Drugs on Cell Proliferation Related to Cutaneous Tissue Repair

The effect of the extracts on fibroblast, endothelial cell and keratinocyte proliferation is studied first in order to determine the stimulatory doses and concentration limits for cytotoxicity *in vitro*. Fibroblasts, endothelial cells and keratinocytes are indispensable in cutaneous wound repair. Fibroblasts and endothelial cells play very important roles in the initial phase of wound healing. Fibroblasts migrate into the wound site 24 h after injury. During this phase of healing (4 to 21 days), fibroblasts are activated and undergo a burst of proliferative and synthetic activity, producing high amount of fibronectin, and then synthesising the other protein components of the extracellular matrix, including collagen, elastin and glycosaminoglycans. Fibroblasts also contribute to the contraction of the wound.¹⁶ Endothelial cells play a key role in angiogenesis and critical mediators of the repair reaction. Their proliferation and organisation into vascular network at the wound site, as well as their biosynthetic capacity, are essential for the successful completion of tissue repair.^{16,23} Keratinocytes are very important in re-epithelialisation and wound closure. Keratinocytes are also known to produce a huge amount of cytokines and growth factors as well as adhesion molecules that regulate fibroblast proliferation, angiogenesis and formation of basement membrane zone.¹⁶

Fibroblasts, endothelial cells or keratinocytes are seeded in 96-multiwell plates at density of 1×10^3 cells/well for fibroblasts and endothelial cells or 2×10^3 cells/well for keratinocytes in growth media and incubated at 37°C in a 5% CO₂/95% air atmosphere for 24 h or 48 h. In this condition, cells recover from trypsinisation subculture or thawing. After 24 h or 48 h, cells are switched to basal media for another 48 h. Then various concentrations of the plant extract diluted in basal media are added to cells. Two sets of cells fed with basal or growth media without addition of the extract are considered as negative or positive control, respectively. Cell growth was estimated by MTT assay at different time intervals.

In our previous study on the aqueous extract from the

leaves of *Chromolaena odorata*, enhanced growth of fibroblasts and endothelial cells was found at concentrations of 10 µg/mL and 100 µg/mL.²⁴ The extract at concentrations of 0.1 µg/mL to 5 µg/mL significantly stimulated human keratinocyte growth in monolayer as they did in growth medium. The cells tended to clump together and differentiate when the doses of the extract were increased from 50 µg/mL to 300 µg/mL.²⁵ In another study on polyphenolic compounds of *Cudrania cochinchinensis* using the same method, a range of low concentrations from 0.1 µg/mL to 5 µg/mL was shown to stimulate human dermal fibroblast growth.²⁶

Study of Antioxidant Effects of Plant-based Drugs In vitro

The formation of new tissue and the protection of normal tissue from destruction caused by various components of the inflammatory process such as reactive oxygen species occur during wound repair. In the early phase of wound healing, the inflammatory cells and injured tissue produce free radicals which could help host defense against bacteria invasion. This response may be overwhelmed leading to impairment of tissue repair. In the burn wound, free radicals have been found to be responsible for progressive tissue injury and further necrosis. It has been postulated that oxidants contributed to the cellular damage in the zone of stasis. That is a reason why this zone will be necrotic and eventually converted into the deeper wound requiring skin graft.^{27,28} In the chronic wound like venous leg ulcers, the ischaemia and tissue hypoxia associated with the development of venous ulceration can generate oxygen-derived free radicals by converting the enzyme xanthine dehydrogenase to xanthine oxidase which catalysed the conversion of oxygen to the superoxide anion. This free radical is very toxic to tissues and may also result in the generation of free radicals of other types. When a tissue is reperfused following a period of ischaemia, the new supply of oxygen in the returning blood supply permits large amounts of the superoxide anion to be produced.²⁹ It is well documented that the topical application of compounds with free radicals scavenging properties on wounds have obviously shown that antioxidant agents significantly improved wound healing and protected cutaneous tissue against oxidative damage.³⁰⁻³³ Many plant extracts and medicinal herbs have shown potent antioxidant activity and flavonoids, the main components of plant extracts act as powerful free radical scavengers.^{34,35}

Some basic research of the role of antioxidants from plant extracts in wound healing has been well published.³⁶⁻³⁹ Again, cultured fibroblasts, endothelial cells and keratinocytes are employed in *in vitro* experiments to assess the protective effects of the extracts on cells against oxidative induced damage. Strong oxidant agents, commercial hydrogen peroxide (H₂O₂) or hypoxanthine-

xanthine oxidase, are prepared to add to cells. Cells are seeded in 96-well plates at high densities of 6,000 cells/well or 10,000 cells/well in growth media, respectively and incubated at 37°C in a 5% CO₂/95% air atmosphere. Cells are grown to near confluence. To investigate the protective or scavenging activities of plant extracts, cells are washed with buffer solution and different concentrations of the extracts and hydrogen peroxide (H₂O₂) or hypoxanthine-xanthine oxidase in serum-free media are simultaneously added to cells. The controls are normal cell culture without addition of extracts or oxidant agents and cells exposed to oxidative damage without presence of extracts. Cells are then incubated for another three hours, washed twice with buffer solution, and fresh serum-free media are replaced to the wells. The MTT assay is used for the assessment of both cell damage and the effects of the extracts on the extent of oxidative damage.

We also used this technique to study antioxidant effects of wound healing extracts from *Chromolaena odorata*. The results showed that the toxicity of hydrogen peroxide or hypoxanthine xanthine oxidase to fibroblasts was a dose-dependent relationship. The total ethanol extract at 400 µg/mL and 800 µg/mL always showed maximum and consistent effect on oxidants in low or high doses. To keratinocytes, the dose-dependent relationship of oxidant toxicity was only seen in hydrogen peroxide but the protective degree of the extract at certain concentrations obviously depended on the oxidant doses. The total ethanol extract at 400 µg/mL or 800 µg/mL showed fully or half effect on low or high concentration of oxidants (Fig. 1).⁴⁰ In the study to deal with polyphenolic substances from *Cudrania cochinchinensis*, the strong antioxidant activities of the extract on fibroblasts and endothelial cells were claimed at concentrations of 5 µg/mL or 50 µg/mL.^{26,41}

Study of Effects of Plant-Based Medicines on In vitro Model of Wound Contraction

Wound contraction is a basic biological repair process necessary for the closure of open wounds. However, in wound like burn injuries, wound contraction may be excessive and lead to scar contracture producing both functional and cosmetic deformities. Both of these contractures can lead to not only physical impairment in healing but also psychological problems. Some of these can be horrendous.⁴² It is very important in the treatment of burn wounds and skin injuries to minimise scar contracture and deformities. To this end, the approach has been to use topical pharmacological agents as well as pressure garments. However, the latter method is very difficult for patients to comply with particularly in countries that have hot, tropical climates. An *in vitro* model for wound contraction study uses fibroblast-populated collagen lattices (FPCL) in which the effects of the plant extract can be investigated. Collagen

gels are prepared from type I collagen extracted from rat tail tendon in 0.1% acetic acid. Working solutions are made by diluting the collagen to 1-2 mg/mL from the concentrated stock solution. Normal dermal fibroblasts, hypertrophic or keloid-derived fibroblasts are cultured from normal skin or scars using explant method. Cells are maintained in optimal culture condition. Low passages of fibroblasts should be employed for the experiments. Cells are trypsinised, resuspended in growth medium and counted using a hemocytometer. The volume of suspension is adjusted to give a cell density of 3.4×10^5 cells/mL and placed on ice at 0°C while the collagen polymerising solution is made. The cell suspension is uniformly distributed in the collagen polymerising solution, giving a density of 6.7×10^4 cells/mL. 2 mL of the collagen/cell suspension is dispensed into 32 mm diameter non-tissue culture treated dishes and the lattices are incubated for 20 min at 37°C in a 5% CO₂/95% air atmosphere. Then 1 mL of medium either with or without additions of various concentrations of the extracts is added to the FPCLs. The gels are released from the sides and bottom of the dish with a spatula and this is considered as zero time. The lattice diameters are assessed at various time points for several days by placing the dish on transparent graph paper and measuring 2 diameters at right angles to one another. The area is calculated from the mean of the diameters and expressed as a percentage of initial lattice area (Fig. 2). For visualisation of living fibroblasts in lattice, MTT assay is used. At the end of the experiment, the medium is removed from the dishes and 2 mL Hanks' balanced salt solution without phenol red is replaced to each dish. Then 200 µl of the tetrazolium salt MTT is added to permit the visualisation of living cells in the lattices. The mitochondria of living cells reduced the MTT to a blue formazan product. After 2 h, the lattices are observed under a light microscope. Photomicrographs are taken.⁴³

The significant inhibition of collagen gel contraction by the Eupolin extract at 50 µg/mL to 200 µg/mL is demonstrated in various concentrations of collagen. When the extract at 50 µg/mL to 150 µg/mL was washed out of the lattices and replaced by fresh medium without Eupolin, the contraction of collagen by cells was resumed. The visualisation of cells in the lattices by incubation in the 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide MTT for 2 hours showed live cells at 50 µg/mL to 150 µg/mL of the extracts. In contrast, all cells were killed at higher extract doses of 300 µg/mL or 400 µg/mL.⁴³

Study of Effects of Plant-Based Medicines on In vitro Model of Wound Re-epithelialisation

Until the wound is re-surfaced by the epithelium, and the protective stratum corneum is re-established, the patient remains at risk of infection, and continues to suffer losses of water, heat and nutrient from the wound surface.



Fig. 1a.



Fig. 1b.

Fig. 1. Protective effects of the total ethanol extract from *Chromolaena odorata* on fibroblasts and keratinocytes against hydrogen peroxide induced damage. Human dermal fibroblasts (a) and epidermal keratinocytes (b) in confluent monolayer monitored under the light microscope in optimal cell culture condition (x100). Cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to visualise living cells. This photograph shows healthy living cells took up MTT and formed blue formazan in cytoplasm.



Fig. 1c.



Fig. 1d.

Cytotoxicity of hydrogen peroxide at concentration of 2×10^{-4} mol/L to fibroblasts (c) and to keratinocytes (d) in confluent monolayer monitored under the light microscope in serum-free medium without the addition of extract (x100). Cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to visualise living cells. This photograph shows cells were killed off by the oxidant and washed off from the tissue culture plastic.

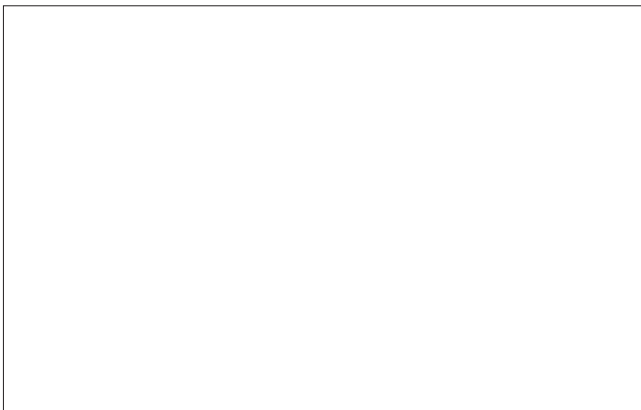


Fig. 1e.

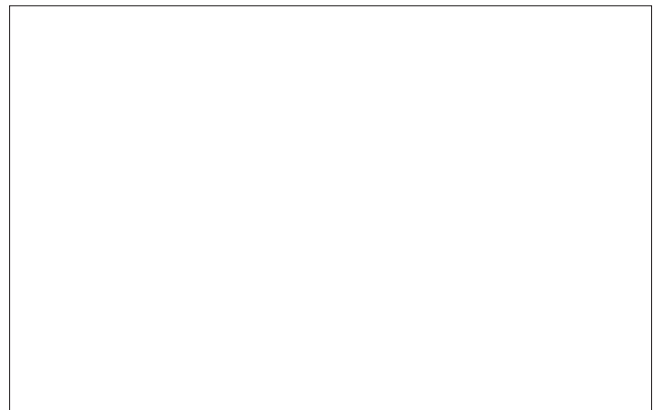


Fig. 1f.

Protective effect of the extract on hydrogen peroxide-induced damage to fibroblasts (e) and keratinocytes (f) in confluent monolayer monitored under the light microscope. Cells were simultaneously exposed to hydrogen peroxide at concentration of 2×10^{-4} mol/L and the extract at 800 µg/mL in serum-free medium (x100). Cells were stained with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) to visualise living cells. This photograph shows surviving cells under extract protection against oxidative damage took up MTT and formed blue formazan.

Understanding the way in which re-epithelialisation occurs is therefore, one of the most important aspects of wound healing. Re-epithelialisation involves both locomotion and

proliferation of keratinocytes remaining in or around the wound site. The most important sources of keratinocytes are the remaining hair follicles, sebaceous and sweat

glands or from the wound edge.^{44,45}

There are several methods to assay the migration and re-epithelialisation of human keratinocytes such as modified Albrecht-Buehler method,⁴⁶ organotypic culture model⁴⁷ or agarose gel keratinocyte outgrowth system.⁴⁸ In our laboratory, scratch technique is generally used as a model of wound re-epithelialisation. Human epidermal keratinocytes, either the second passage or third passage, are seeded at high density from 5×10^5 to 8×10^5 cells/dish in low calcium serum-free growth medium in 32 mm tissue culture dishes. Cells are fed every 2 days until they reach 100% confluence. Prior to wounding, high calcium and serum medium is replaced for 4 hours. In this condition, the formation of desmosomes between neighbouring cells and strength of cell to cell attachment are established⁴⁹ and reproducible, distinct and straight scratches are made. Each cell layer is scratched using a sharpened plastic micropipette tip. A glass slide is placed across the top of the dish to facilitate steadying of the tip whilst scratching and to enable a straight scratch to be made. The tip is drawn firmly across the diameter of the dish. Several sharpened micropipette tips have to be prepared over the course of the experiment as the plastic tip becomes deformed after several scratches. Cells are washed with PBS in order to remove loosened debris. A range of concentrations of the plant extracts diluted in basal medium is added to a set of 2 or 3 dishes for each dose. One set is fed with basal medium only as control (Fig. 3).

A fibreoptic camera linked to a VDU monitor and a video recorder is attached to the inverted microscope (Leitz Diavert) using a C-mount with a 0.63 lens so that the size of the image on the monitor corresponds to that in the field of the microscope (Fig. 4). For each scratch 4 or 6 consecutive fields are selected using the following criteria: relatively little cell debris within the scratch; even scratch, with straight edges; both edges visible under a single field using the 10X objective; field not too close to either end of the scratch, where distortions uncharacteristic of the main part of the scratch could be seen to occur during the healing process. In order to ensure that the same fields are observed during consecutive measurements, the following techniques are used: The dish is oriented by lining up dots drawn on the bottom of the dish with those drawn on the bottom of a 6-well plate lid (used as a support tray) so that the scratch appeared vertical in the field of the microscope. The dish is fixed on the plate lid using Super Glue. The plate is located on the microscope stage using the horizontal and vertical vernier scale attachments. The coordinates on the vernier scales are noted for each field. It is calculated that on the horizontal vernier scale 1 mm (± 0.1 mm) is the equivalent length of one field. Hence, to advance the plate by one field it is moved 1 mm on the vernier scale. The condition of each field is recorded on video tape at various

intervals over periods up to 72 h after the scratches have been made. Photographs of each field are also taken at the same time points. Changes in the denuded area are monitored using the Microscan system (Fiber Optics Research). The Microscan is calibrated in the X and Y directions for the 10X objective using a microscope stage graticule of 1mm length with 0.01 mm subdivisions. A roll ball tracker is used to outline the denuded area in the particular field and the size of this is calculated by the Microscan software. The precision of this measurement is estimated to be ± 0.001 mm² per field. The percentage of the initial area still denuded is calculated for each of the 4 or 6 selected fields and averaged for each recording time. The average of two dishes is then taken for each concentration of the extract and for each control.

With this model, the extract from the leaves of *Chromolaena odorata* was investigated. It was shown that concentrations of the extract of 5 µg/mL to 60 µg/mL always led to faster migration of keratinocytes and closure of the scratch, although there was no clear dose-dependent relationship.²⁵ Using similar technique, Zimmer et al⁵⁰ had demonstrated the enhancement of keratinocyte migration by hydrophilic and lipophilic extracts from *Gryllotalpa africana*, a Traditional Chinese Medicine using mole crickets for treatment of problematic wound.

Study of Effects of Plant-Based Medicines on Adhesion Molecule Production by Keratinocytes In vitro

Extracellular matrix (ECM) and basement membrane zone (BMZ) components as “adhesion” molecules serve several critical functions for effective wound repair and are crucial to basal cell regeneration and wound re-epithelialisation. BMZ components serve as a scaffold for tissue organisation and as a template for tissue repair. These components are produced and regulated by keratinocytes, fibroblasts and cytokines.⁵¹ In a previous study, we used an indirect immunofluorescent technique to investigate the effects of increasing doses of the extract on the expression of the adhesion complex proteins of the skin by cultured human keratinocytes.

Cells are seeded onto multi-well slides and grown in growth media for 48 h. Cells are switched to basal medium for another 48 or 72 h. Basal medium supplemented with increasing doses of extract are replaced. The cells are harvested, washed in PBS, air dried and stored at -70°C until required. The cultured keratinocytes are probed with monoclonal antibodies to keratin, desmosome components, integrins, laminins, collagen type IV, collagen type VII, and fibronectin. Normal intact skin served as the tissue control, L-15 tissue control medium, ascites non-immune serum, and anti-tenasin served as negative controls.

We found enhanced expression and secretion of laminin

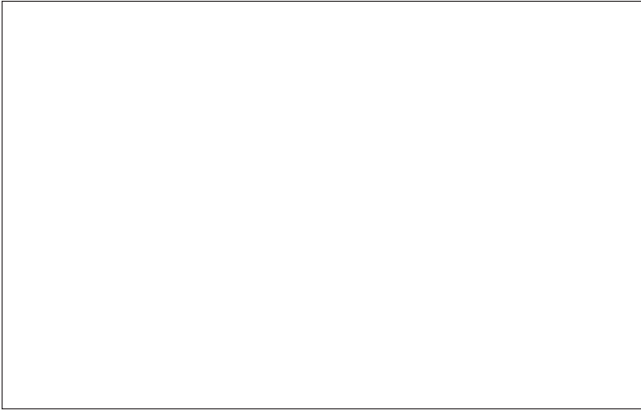


Fig. 2a. Contraction of fibroblast-populated collagen lattices in serum medium.

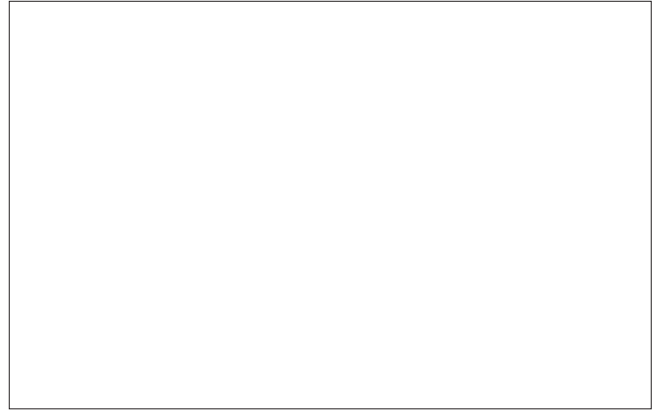


Fig. 2b. Photomicrograph of human dermal fibroblasts populated in collagen gel (x200).



Fig. 3a.



Fig. 3b.



Fig. 3c.

Fig. 3. Photomicrographs of an in vitro wound assay made by scratching on confluent monolayer of human epidermal keratinocytes and cell migration into the "wound" over the period of time. Arrows show the denuded areas: (a) at 0 hour, (b) at 32 hours and (c) at 64 hours.



Fig. 4. The instrument for monitoring keratinocyte migration in an in vitro wound model —A fiberoptic camera linked to a VDU monitor and a video recorder is attached to the inverted microscope (Leitz Diavert).

5, collagen IV, fibronectin, demoplaikin, keratin 16, and laminin by the aqueous extract of *Chromolaena odorata*. This expression was dose-dependent. The expression of $\beta 1$ and $\beta 4$ integrins was also up-regulated by the extract concentrations at 0.1 $\mu\text{g/mL}$ and 1 $\mu\text{g/mL}$ and that seemed to be decreased with increasing doses. Keratin (general) was expressed at all doses. The extract had no effect on the

expression of the $\alpha 6$ intergrin, and only minimal effect on the expression of keratin 1, 10 and desmoglein.⁵²

The positive findings from immunofluorescence data can be reconfirmed by Western blots or measured gene expression using Northern blots.

In vitro Model of Angiogenesis

Angiogenesis, the formation of new vessels during

development in response to injury, is a dynamic process. The principle cell type involved in the process of angiogenesis is the microvascular endothelial cell. This phenomenon is important in granulation tissue formation and modulated by other factors and cells involved in the healing process.⁵³

The *in vitro* model of angiogenesis was developed by Nicosia et al⁵⁴ as a quantitative assay of neovascularisation *in vitro*. Angiogenesis is studied by culturing rings of aorta in three-dimensional matrix gels in the presence of serum-free medium. Aortic rings are embedded in gels of fibrin or interstitial collagen. Each gel is prepared in a cylindrical well obtained by placing an agarose ring on the bottom of a culture dish. Newly formed microvessels in the living cultures are counted daily. Curves of microvascular growth are obtained by recording the increase in the number of microvessels over time. The microvascular nature of the outgrowths is confirmed by immunostaining of factor VIII-related antigen and by ultra-structural analysis.

Effects of plant extracts on wound healing on *in vitro* angiogenesis can be investigated using the same model.

In vitro Model of Cultured Living Skin Equivalent (Organotypic Culture Model)

Wound repair is a complex biological process and controlled by means of cell-cell contacts, by the production of mediators and adhesion molecules and by their interaction with the surrounding extracellular matrix. The development of the cultured living skin equivalent has provided another appropriate tool to study the pharmacological effects of plant-based medicines on three-dimensional cell culture and more *in vivo* equivalent condition.

Living skin equivalents are formed by seeding human epidermal keratinocytes onto the upper surface of fibroblast-populated collagen lattices and subsequently maintained at the air-liquid interface for 10 days to allow the terminal differentiation of epidermal layers. Cultured skins are wounded by complete bisection and the two semi-circles are transferred to an acellular collagen gel.^{55,56} The various concentrations of plant extracts are added and maintained for a period of time. Samples are frozen, sectioned and subjected to immunostaining analysis for specific protein expression during re-epithelialisation.

Conclusions

Wound healing involves several overlapping phases: inflammation, formation of granulation tissue, re-epithelialisation, scar formation and tissue remodelling. Cultured cells permit the use of a number of simple models to study certain aspects of the healing process in isolation, without the complication of other factors. They also allow investigation on the influence of pharmacological agents

which can modulate the process. In this article, we review in detail some *in vitro* cell culture models that have been utilised and developed in our institutions for investigation of plant extracts for wound healing. In summary, traditional medicine offers a wealth of knowledge that can be practiced in the routine management of wounds and, when investigated scientifically, can lead to information on their mechanism that will enhance our understanding of how the agents affect the wound healing process. Clinical efficacy of traditional agents should be based on proper prospective, randomised, controlled trials.

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