Simple Evaluation On Wound Healing Properties Of *Lygodium Japonicum* Leaf Extract

by

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CERTIFICATE

This is to certify that the dissertation entitled

"Simple Evaluation On Wound Healing Properties Of *Lygodium Japonicum* Leaf Extract"

is the bonafide record of research work done by

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During the period from August 2005 to April 2006 under our supervision

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ABSTRAK

Penilaian ringkas ke atas aktiviti penyembuhan luka ke atas tikus strain Sprague-Dawley dilakukan menggunakan penyediaan ekstrak kasar daun *Lygodium japonicum*. Ekstrak kasar tersebut (50mg) dirawat pada permukaan luka secara sekali (1) kali rawatan, tiga (3) kali rawatan secara berturut-turut manakala tikus kawalan tanpa rawatan ekstrak. Kontraksi dan kekuatan tensil diukur untuk menilai kesan ekstrak *Lygodium japonicum* ke atas penyembuhan luka. Keputusan kajian menunjukkan bahawa ekstrak kasar daripada daun *Lygodium japonicum* tidak memberikan kesan kontraksi luka dan kekuatan tensil yang signifikan berbanding kajian kawalan.

ABSTRACT

Simple evaluation on the wound healing activities on Sprague-Dawley rats using the preparation of crude leaf extract of *Lygodium japonicum* was done. The crude extract (50 mg) was applied to the surface of wounds ones, three (3) consecutive times, whereas for control rats were not treated at all. The contraction and tensile strength were measured to evaluate the effects of *Lygodium japonicum* on wound healing. The results showed no significant difference in wound constraction and tensile strength using the *Lygodium japonicum* crude leaf extract compared to control experiment.

INTRODUCTION

1.1 Background research

The application of medicinal concoctions from plants to treat skin lesions, in particular, burns and wounds, has had a long tradition. There are many drugs derived from the plant used in the treatment for diseases like cancer, diabetes, rheumatism, hypertension, aphrodisiac, anti-microbial, wound healing, and etc. In spite of the tremendous advances in the chemical drug industry, the availability of substances capable of stimulating the process of wound repair is still limited (Udupa *et al.*, 1995). Plants with wound healing activity have been reported and experimentally studied on various animal models to reveal the most promising active compounds (Abu-Al-Basal, 2001). Wound healing is becoming a crucial health issue in the world and is focused mostly in the develop country. The wound healing process of the skin occurs naturally in our body. However, wound healing process can be vulnerable to infections, contaminations or other complications. Recently, the synthetic drugs used in the treatment of wound are found to cause allergic reaction to the patient and also cause microbial resistance to the drugs. Thus, the discovery of new drugs derived from plants will provide an alternative way in treating wound and wound healing process.

Lygodium japonicum or japanese climbing fern is one of the many plants used by the Malays as a treatment in herbal medicine. It is believed that the leaves of the plant have great potential in inducing wound healing activity as well as in reducing the sense of wound pain. "Pokok Seribu" as is it known locally, is used in treating wounds derived from knife or by accident. This plant however has not been extensively studied for its wound healing properties. However, it is important to scientifically study the plant for the presence of valuable compounds that may be useful in the treatment of wounds.

1.2 *Lygodium Japonicum*

Lygodium japonicum or climbing fern grows wild in areas from Japan to the Himalayas and down to Northern Australia. In Malaysia it is known as "Pokok Seribu" and is widely used locally by traditional medical practitioners to treat various minor cuts and wounds. It belongs to the family Lygodiaceae. It has twinning, threadlike stems with pinnate papery fronds. The stem is slender but difficult to break. This plant will reach a maximum height of about 8 feets (2.5m) with the fronds reaching to about 4-8 inches (10-20 cm) in length. The fertile fronds which are usually smaller segments with fingerlike projections around the margins bearing spore. The sterile pinna consists of a lobed segment called pinnules. The fertile pinnea is narrow and 3 times divided (anonymous).

Lygodium japonicum needs light to heavy shade with a moist well drained soil mix. They are propagated by binary division or by spores. The mature spores can be collected on a piece of paper placed under the spore-bearing leaves.

Figure 1.1: A photographed of the *Lygodium japonicum* shown in finger like shape.

1.2.1 Ethnopharmacological activities

In Japan, scientists from Kinki University and Daiichi Pharmaceutical company have found the liphophilic constituents of *Lygodium japonicum* as an anti-androgenic and hair growth promoting activities. The aqueous ethanol extract of lygodii spore (spore of *Lygodium japonicum*) showed *in vitro testosterone 5 a-reductase* inhibitory activity and *in vivo,* and as anti-androgenic activity using growth of flank organ in castrated Syrian hamsters hair regrowth after shaving with testosterone-treated C57Black/6CrSlc mice. The major lipophilic constituents of lygodii spore are oleic, linoleic and palmitic acids which were identified as the main active principles inhibiting the *testosterone 5 a-reductase.*

Lygodium japonicum has also been widely used in the traditional chinese medicine in the treatment of patient with gallstone (Dharmananda *et al.,* 2001). This species has been used as one of the ingredients in a formulation called 'Three Gold Formula' having the properties to dissolve gallstone (Hson *et al.*, 1986). *Lygodium japonicum* is also used in the urinary stones treatment in persons with damp-heat syndrome and urinary retention (Yan Wu *et al.*, 1997). It was used to help remove urinary stones in urinary bladder, ureter or urethra. The three golds may be added to any traditional formula for the treatment of urinary blockage when stones are diagnosed. It is produced in two forms, one in the decoction and the other is the powder form. A typical recommendation for daily dosage is 6-12 grams in the decoction, or 2-3 grams in the powder form.

1.2.2 Phytochemical studies

The chemistry of the *Lygodium japonicum* has not been extensively studied and only a few of the chemical constituents of the plant have been extracted, isolated and identified. In a study of *Lygodium japonicum* as anti-androgenic and hair growth promoting activities, the fatty acids or the lipophilic constituents of lygodii spore of *Lygodium japonicum*, such as oleic, linoleic and palmitic acids were isolated in trace quantities from the culture medium of *Lygodium japonicum* prothallia (Hideaki *et al.*, 2001). Researches have found out that this plant, when combined with several chemical substances, will supply minerals and vitamin to patients having kidney problems (Dept. of Health and Human Services, FDA, 2000). *Lygodium japonicum* also contained chemical substances that are used to treat damp-heat syndrome with urinary retention, and to remove urinary stone (Dharmananda *et al*, 2001).

Even though that the plant has been used in the treatment of the above ailment, its used in the treatment of wounds have not been studied. Therefore, it is imperative to study the plant wound healing properties so that its traditional used in wound treatment can be scientifically proven.

1.3 Wound healing process

Wounds can be described in terms of physical injuries that results in an opening or break of the skin or disruption of the anatomic structure and its function in any part of the body. Therefore, healing is the mechanism in which involving the restoration of that structure and function. Wound healing is a complex and dynamic sequence of events which involve four phases (Ross, 1980 and Baie & Sheikh, 2000):

I. *Coagulation*, which prevents blood loss.
II. Inflammation and debridement of wound.
III. *Epitheliar repair*, including proliferation, mobilization, migration and differentiation.
IV. Tissue remodeling and collagen deposition

There are several categories of wound healing have been described such as primary healing, delayed primary healing, healing by secondary intention and, healing of partial-thickness wounds. Even though different categories exist, the interactions of cellular and extracellular constituents are similar. Primary wound healing or healing by first intention occurs within a few hours of its creation. Wound edges are surgically or mechanically approximated, and collagen metabolism provides long-term strength. If the wound edges are not reapproximated immediately, delayed primary wound healing transpires. This type of healing may be desired in the case of contaminated wounds.

A third type of healing is known as secondary healing or healing by secondary intention. In this type of healing, a full-thickness wound is allowed to close and heal. Secondary healing results in an inflammatory response that is more intense than with primary wound healing. In addition, a larger quantity of granulomatous tissue is fabricated because of the need for wound closure. A fourth category is healing that transpires with wounds that are only partial skin thickness (Glat *et al.*, 1997). Healing of partial-thickness wounds occurs when a partial-thickness wound is closed primarily by epithelialization. This wound healing involves the superficial portion of the dermis with minimal collagen deposition, and an absence of wound contraction.

Any agent which accelerates the above processes is a promoter of wound healing. The process of wound healing differs little from one kind of tissue to another and is generally independent of the form of injury. Although the different aspects of the wound healing process occur in a continous, integrated and complex manner, it is more convenient to divide the overall process into three overlapped phases and several natural components for descriptive purposes, as in the diagram below. Patologically, wound healing comprises of an important sequence of inflammation and repair (Fishman *et al.*, 2004). Basically the epithelial endothelial cells, inflammatory cells, platelets, and fibroblast come together at the side of the bleeding, and interact among each other to restore the tissue normal function around that region. The diagram shows that there are two bell-shaped graphs that represents two important overlapped process involved in healing process of the wound.

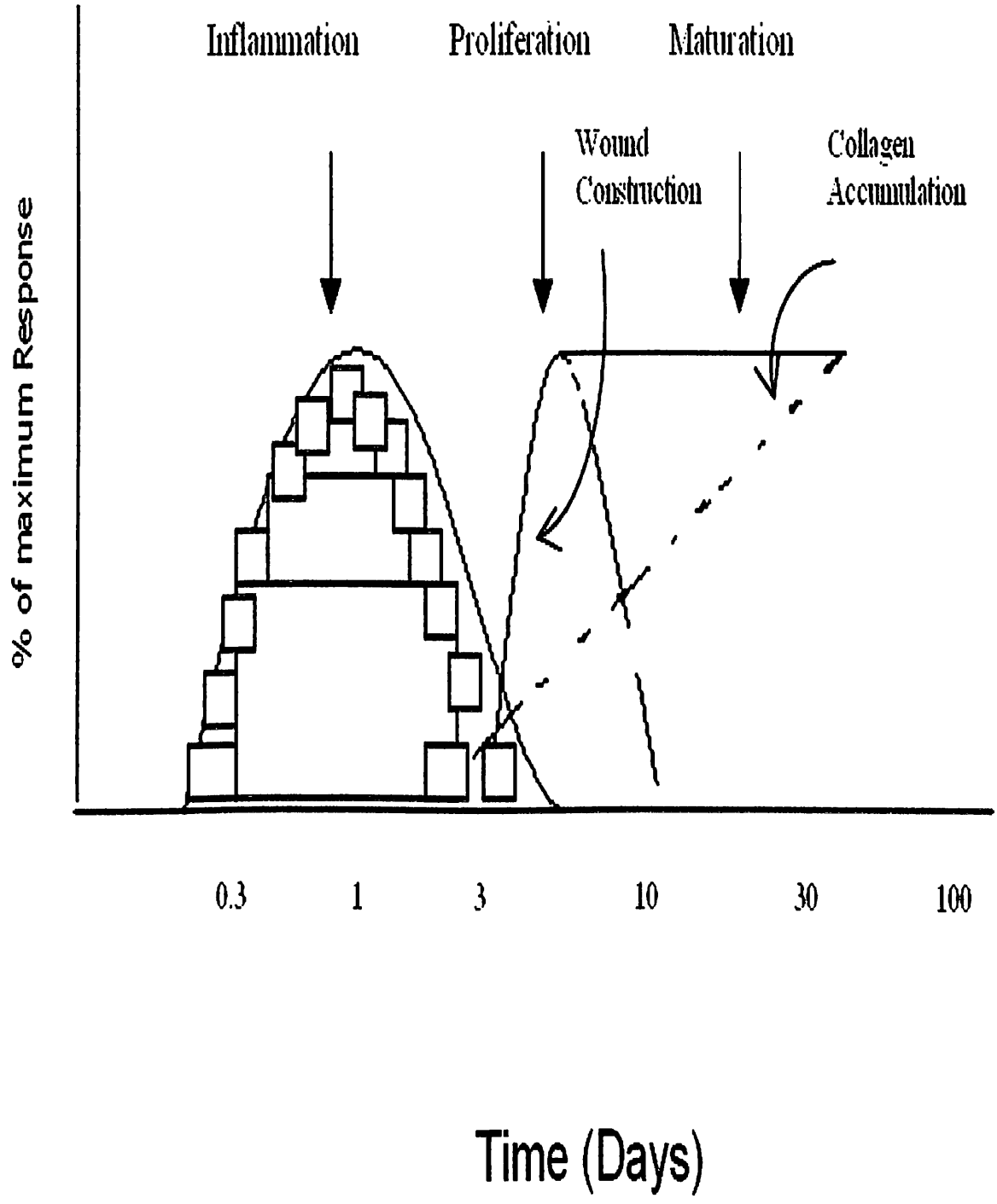

Figure 1.2: Wound Healing Phases

1.3.1 Inflammatory phase (0-5 days)

The healing response is initiated at the time of injury. Hemmorhage will follows, that usually caused by surgical or traumatic cut of the skin. Transient vasoconstriction and coagulation followed by dilation and capillary leak of plasma to the wound area. Coagulation, as mentioned, is often called the first stage of healing because of its primary importance in this process. Platelets provide the initial signals to begin the repair process. Alpha granules of the platelets produce growth factors (PDGF, TGF-β, Platelet factor IV), which initiate the wound healing cascade by attracting and activating fibroblasts, endothelial cells, and macrophages. While, dense bodies of the platelets store vasoactive amines (serotonin) which increase vascular permeability.

Fibroblasts are attracted to the wound and stimulated to proliferate and transforming growth factor beta (TGF-β) to induce collagen production by platelet derived growth factor. In the early inflammation, complements are activated and inflammatory cytokines are derived from fibroblasts. Vasodilation, pain and swelling is seen due to the production of kinins and prostaglandin. Fibrocytes, probably travel from blood to the wound to form fibroblasts but may also arise from adjacent tissue. Within 6 hours after injury, neutrophil or also known as polymorphonuclear leucocytes (PMN), the first blood leucocytes to come to the wound site are peak at 24-48 hours.

1.3.2 Proliferative phase (day 3-14)

As mentioned, neutrophils predominate initially at the side of injury but later, the neutrophils die off and macrophages become the predominat cell type in the wound. These cells are derived from circulating monocytes by a combination of a migration and chemotaxis by attractants such as complements, IgG fragments, collagen, cytokines, platelet factor IV, and platelet growth factors (PDGF & TGF-β). They first appear within 48-96 hours post-injury and reach a peak on the third day post-injury. These macrophages have a much longer life span than the PMN and persist in the wound until healing is complete. Their peak numbers occur about the seventh day after injury. The presence and activation of both macrophage and lymphocytes in the wound is important to the progress of the normal healing process.

Macrophages are phagocytic and are the primary producer of the growth factors responsible for proliferation of extracellular matrix, smooth muscle, and endothelial cells resulting in angiogenesis. These cells are just like neutrophils and phagocytes and digest pathological organism and tissue debris. In addition, macrophage releases biologically active substances. Many of these substances facilitate the recruitment of additional inflammatory cells and help macrophage in tissue decontamination. In addition, growth factors and other substances are also released which are nessesary for the initiation and propagation of granulation tissue formation. These intercellular transmitters are known as cytokines.

Granulation tissue consists of cellular elements, including fibroblasts and inflammatory cells, along with new formation of blood capillaries in a loose extra cellular matrix of collagen, fibronectin and hyaluric acid. Fibroblasts first appear in significant numbers in the wound on the third day post-injury and achieve peaks around the seventh day. Their major role is to synthesis of an ECM comprising primarily collagen. Whereas fibroblasts deposit type III collagen as part of the provisional matrix early in the wound repair, and their subsequent secretion of type I collagen that provides a structural support for healing. This rapid expansion in the fibroblast population at the wound site occurs via a combination of proliferation and migration.

Fibroblast are derived from local mesenchymal cells, particularly those associated with blood vessel adventitia, which are produced proliferate and attracted into the wound by a combination of cytokines produced initially by platelets and subsequently by macrophages and lymphocytes. Fibroblasts are the primary synthetic element in the repair process and are responsible for the production of the majority of structural proteins used during tissue reconstruction. Basically, fibroblasts produce large quantities of collagens, which form the main constituent of the extracellular wound matrix and ultimately responsible for producing tensile strength to the scar. Collagens are detected in the wound on the third day post-injury, and the levels increase rapidly for approximately 3 weeks. It then continues to accumulate for up to 3 months.

The collagens are initially deposited in haphazard fashion and these individual collagens fibrils are subsequently recognized by cross-linking, into regularly aligned bundles oriented along the lines of stress in the wound healing. Fibroblasts are also responsible for the production of other matrix constituents including fibronectin, hyaluric acid and the glycosaminoglycans. The process of fibroblasts proliferation and synthetic activity is known as fibroplasias. Revascularization of the wound proceeds afterwards. Capillary buds sprout from blood vessels adjacent to the wound and extent into the wound space. Endothelial cells from the side of the venule closests to the wound begin to migrate in response to angiogenic stimuli, at the second day post-injury. These capillary sprouts eventually branch at their tips and join to form capillary loops which blood begins to flow.

Angiogenesis occurs by a combination of proliferation and migration. Cytokines are produced by platelets macrophages and lymphocytes in the wound. The potential cytokines mediators of neovascularization are basic fibroblasts growth factor (bFGF), acidic FGF (aFGF), transforming growth factors-α and β (TGF-α and–β) and epidermal growth factor (EGF) have been shown to be potent stimuli for new vessel formation. Restoration of epithelial integrity takes place at the wound surface. Repithelialisation of the wound begins with a few hours of the injury. Epithelial cells, arising from either the wound margins or residual dermal epithelial appendages within the wound bed, begin to migrate under the scab and over the underlying viable connective tissue.

The epidermis immediately adjacent to the wound edge begins thickening within 24 hour after injury. Marginal basal cells at the edge of the wound loose their firm attachment to the underlying dermis, become enlarged and begin to migrate across the surface of the provisional matrix filling the wound. Fixed basal cells in a zone near the cut edge undergo a series of rapid mitotic divisions, and these cells appear to migrate by moving over one another in a leapfrog fashion until the defect is covered. Once the defect is bridged, the migrating epithelial cells loose their flattened appearance become more columnar in shape and increase in mitotic activity.

Layering of the epithelium is re-established and the surface layer eventually keratinized. Reepithelialization is complete in less than 48 hour in the case of approximated incised wounds, but may take substantially longer where there is a significant tissue defect. If only the epithelium is damaged, such as occurs in split thickness skin graft donor sites, then repair consists primarily of repithelialization with minimal or absent fibroplasias and granulation tissue formation. The stimuli for repithelialization remain incompletely determined, but it appears that the process is mediated by acombination of loss of contact inhibition, exposure of constituents of the extracellular matrix particularly fibronectin, and by cytokines produced by immune mononuclear cells, EGF, TGF-β, bFGF, platelet derived growth factor (PDGF) and insulin like growth factor-1 (IGF-1) in particular, have been shown to promote epithelialization.

1.3.3 Maturation phase (day 7 to 1 year)

Iniatilly, the extracellular is rich in fibronectin. Fibronectin forms a fibre network. There are also presences of significant quantities of hyaluronic acid and large molecular weight proteoglycans, which contribute to the gel-like consistency of the extracellular matrix and help cellular infiltration. Collagen becomes the predominant constituent of the matrix. Randomly distributed collagen fibres become cross-linked and aggregated into fibrillar bundles, which gradually provide the healing tissue and increasing tensile strength and stiffness. After a 5-day lag period, there is a rapid increase in wound breaking strength due to collagen fibrogenesis. The subsequent rate gain in wound tensile strength is slow, with the wound having gained only 20% of its final strength after 3 weeks. The final strength of the wound remains less than that of uninjured skin, with the maximum breaking strength of their scar reaching only 70% of that of the intact skin.

This gradual gain in tensile strength is not only due to continuing collagen deposition, but also to collagen remodeling, with alteration of inter molecular crosslinking and formation of larger collagen bundles. Collagen remodeling during scar formation is dependent on both continued collagen synthesis and collagen catabolism. The degradation of wound collagen is controlled by a variety of collagenase enzymes. The net increase in wound collagen is determined by the balance of this opposing mechanism. The high rate of collagen synthesis within the