UNIVERSITI PUTRA MALAYSIA

EFFECTS OF TOPICAL APPLICATION OF MIXTURES OF MORINDA CITRIFOLIA L., MELASTOMA MALABATHRICUM L., AND LAWSONIA INERMIS L. ETHANOLIC EXTRACTS ON EXCISION WOUND IN SPRAGUE DAWLEY RATS

ALI KHAIRULLAH ZAHI ALSAEED

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By

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Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfilment of the Requirement for the Degree of Master of Science

January 2015

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Abstract of thesis presented to the senate of Universiti Putra Malaysia in fulfillment of the requirement for the degree of Master of Science

EFFECTS OF TOPICAL APPLICATION OF MIXTURES OF *Morinda citrifolia* L., *Melastoma malabathricum* L., and *Lawsonia inermis* L. ETHANOLIC EXTRACTS ON EXCISION WOUND IN SPRAGUE DAWLEY RATS

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January 2015

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The eligibility of attempting to repair excision wounds has long-term been appreciated since the last century, at least in the sensation of providing a spotless wound, avoiding formalization of purulent and pulverization as well as to boost a granulation and re-epithelisation. It has been a particular vision in medical practice as excision wound denatures cellular protein, inhibits cellular metabolism hence secondary interference of local vascular supply. Therefore, the present study was designed to investigate the effects of the mixture of ethanolic extracts of *Morinda citrifolia* fruits, *Melastoma malabathricum* leaves and *Lawsonia inermis* leaves on the excision wound healing as these natural herbs have been traditionally used in excision wound treatment. Prior to wound healing experiment, the dermal toxicity levels of each herbal extract were determined via dermal toxicity experiments using the Organization for Economic Co-operation and Development (OECD) standard guidelines.

Results showed that the lethal dose 50 (LD_{50}) of each herb was more than 5000 mg/kg body weight, while the no observed adverse effect level (NOAEL) for each extract was more than 2000 mg/kg body weight. Based on the dermal toxicity results, mixtures of the herbal extracts at 0.5% and 1% were selected for treatment in the wound healing study. An area of uniform wound 2 cm in diameter was inflicted on 120 adult male Sprague Dawley weighing between 250-350 g. The animals were divided into five groups with six animals in each group, representing a control and experimental groups. Mixture of the herbs at 0.5%, mixture of the herbs at 1%, silver sulphadiazine as a standard treatment and paraffin were applied once daily, except in the control group where wounds were left without any topical treatment.

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The rats were closely monitored to assess any changes. The rats were euthanized at 4, 8, 12 and 21 days post wounding. The macroscopic appearances of excision wounds were evaluated and recorded. The percentages of wound contraction, wound size and wound epithelisation were measured and analyzed. In addition, histopathological examination of the skin was also performed qualitatively and quantitatively using haematoxylin and eosin (H&E), van Gieson and immunohistochemistry staining methods.

Results obtained from this study revealed that mixture of three herbs at 0.5% showed advanced effects to decrease the period of wound healing process, wound contraction and wound size as compared to the other experimental groups. Quantitative evaluation of the number of inflammatory cells (polymorphonuclear cells and macrophages) from day 4 to day 21 in wounds treated with mixture of herbs at 0.5% demonstrated significant ($p < 0.05$) decrease in number of inflammatory cells from day 4 to day 21, while the number of proliferative cells (fibroblasts) increased from day 8 to day 21. Mixture of the herbs at 0.5% also showed potential to preserve viable dermal tissues and induce a well-formed of angiogenesis with better organisation as compared to the other experimental groups.

On the other hand, the re-epithelisation and level of collagen formation was increased gradually in group that received mixture of herbs at 0.5% compared to the other groups. Qualitative and quantitative evaluation of the expression of vascular endothelial growth factor (VEGF), transforming growth factor alpha (TGF α) and transforming growth factor beta (TGF β) proteins in wounds treated with mixture of herbs at 0.5% and 1% recorded gradual rise in the expression of these three growth factors in wound area. In conclusion, the present study showed that mixture of the three herbs at 0.5% had the most superior treatment agent for excision wounds fallowed by mixture of the herbs at 1% in relation to the macroscopic and histopathological evaluation.

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Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Master Sains

KESAN PENGGUNAAN TOPIKAL CAMPURAN EKSTRAK ETANOL *Morinda citrifolia* L., *Melastoma malabathricum* L., DAN *Lawsonia inermis* L. KE ATAS LUKA EKSISI PADA TIKUS SPRAGUE DAWLEY

Oleh
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Januari 2015

Pengerusi: Hazilawati Binti Hamzah, PhD

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Kesesuaian dalam cubaan untuk merawat luka eksisi telah lama diberi penghargaan semenjak seabad yang lalu, sekurang-kurangnya dari sudut memastikan luka tiada parut, mengelakkan penghasilan dan pengumpulan nanah, dan juga untuk merangsang pembetukan granul dan epitelium. Adalah menjadi satu prinsip dalam amalan perubatan yang mana luka eksisi menyebabkan penguraian protin sel dan merencat metabolisma sel dan menyebabkan gangguan kedua kepada bekalan darah setempat. Oleh itu, kajian ini telah dibuat untuk menyiasat kesan campuran ekstrak etanol buah *Morinda citrifolia*, *Melastoma malabathricum* dan *Lawsonia inermis* ke atas penyumbuhan luka eksisi berikutan herba asli ini telah digunakan secara tradisional dalam rawatan luka eksisi. Sebelum ekperimen penyumbuhan luka dilaksanakan, aras toksisiti kulit bagi setiap ekstrak herba ditentukan dengan mejalankan eksperimen toksisiti kulit berpandukan garis panduan Pertubuhan Kerjasama dan Pembangunan Ekonomi (OECD).

Keputusan menunjukkan bahawa Dos Maut 50 (LD_{50}) bagi setiap herba adalah melebihi 5000 mg/kg berat badan, sementara aras kesan teruk yang tidak nampak (NOAEL) bagi setiap herba ialah melebihi 2000 mg/kg berat badan. Berdasarkan ujian tosisiti kulit ini, campuran tiga jenis herba pada 0.5% dan 1% telah dipilih dalam kajian merawat penyumbuhan luka. Satu kawasan luka samarata bergaris pusat 2 cm telah dibuat pada 120 tikus jantan Sprague Dawley dengan berat badan antara 250-350g. Tikus tersebut dibahagikan kepada lima kumpulan dengan enam ekar tikus dalam setiap kumpulan, mewakili kumpulam kawalan dan rawatan. Campuran herba pada 0.5%. campuran pada 1%, sulfadiazin sulfur sebagai rawatan standard dan paraffin disapukan sekali sehari, kecuali kumpulan rawatan dimana luka dibiarkan tanpa sebarang rawatan setempat.

Tikus tersebut diperhatikan secara dekat untuk menilai sebarang perubahan. Tikus tersebut dimatikan pada hari ke 4, 8, 12 dan 21 selepas dilukakan. Penampilan luka eksisi dinilaikan dan dicatatkan. Peratus kontraksi luka, saiz luka dan luka epithelisation

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pembentukan diukur dan dianalisa. Sebagai tambahan, pemeriksaan histopatologi ke atas kulit dibuat secara kualitatif dan kuantitatif menggunakan kaedah perwarnaan hematosilin dan eosin (H&E), van Gieson dan imunohistokima.

Keputusan yang diperolehi daripada kajian ini menunujukkan campuran ketiga-tiga herba pada 0.5% memberi kesan bagus bagi mengurangkan tempoh masa pemyumbuhan luka., kontraksi luka dan saiz luka berbanding dengan kumpulan eksperimen yang lain. Penilaian kuantitatif ke atas jumlah sel inflamasi (sel polimorfonuklear dan makrofaj) daripada hari ke 4 hingga hari ke 21 dalam rawatan luka menggunakan campuran herba pada 0.5% menunjukkan penurunan seerti ($p < 0.05$) sel inflamasi daripada hari ke 4 hingga hari ke 21, sementara jumlah sel proliferative (fibroblast) meningkat daripada hari ke 8 hingga hari ke 21. Campuran herba pada 0.5% menunjukkan potensi untuk mengutuhkan tisu kulit hidup dan merangsang pembentukkan salur darah baharu yang sempurna dengan orgnisasi yang lebih baik berbanding kumpulan eksperimen yang lain.

Dalam pada itu, pembentukkan semula epitelium dan aras pembentukkan kolagen adalah meningkat secara gradual pada kumpulan yang menerima campuran herba pada 0.5% berbanding kumpulan lain. Penilaian kualitatif dan kuantitatif ke atas ekspresi protin factor tumbesaran endotelial vascular (VEGF), faktor tumbesaran perubahan alpha (TGFα) dan faktor tumbesaran perubahan beta (TGF β) pada luka yang dirawat dengan herba pada 0.5% dan 1% menunjukkan peningkatan secara gradual ekspresi protin bagi ketiga-tiga faktor tumbesaran tersebut pada kawasan luka. Kesimpulannya, kajian ini menunjukkan bahawa campuran ketiga-tiga herba pada 0.5% memberikan agen rawatan yang paling bagus untuk luka eksisi diikuti dengan campuran herba pada 1% berdasarkan penilaian makroskopik dan histopatologi.

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"In the name of Allah, the most beneficent and the most merciful"

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LIST OF ABBREVIATIONS

mm	Micrometer
LDH	Lactate dehydrogenase
ALT	Alanine transaminase
AST	Aspartate transaminase
H&E	Hematoxylin and eosin
SSD	Silver sulphadiazine
G	Gram
^{o}C	Celsius degree
EDTA	Ethylene diamine tetra acetic acid
PCV	Packed cell volume
RBC	Red blood cell
SD	Standard deviation
Cm	Centimetre
WBC	White blood cell
Kg	Kilogram
mg	Milligram

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CHAPTER 1

INTRODUCTION

Wound is a physical injury which leads to breakage of the skin. Wound healing is a natural response to tissue injury which consists of complex and sophisticated cascade of events involving various cellular, molecular and biochemical processes and resulting in the healing of the wound and the restoration of the intact functional barrier (Kondo, 2007). Generally, wound healing process is categorised into three integrating and interfering phases: 1) the inflammatory phase, which is the first phase that consists of the establishment of homeostasis and inflammation, 2) the proliferative phase, which is the second phase that consists of granulation, contraction, and epithelialisation, and 3) the remodelling or maturation phase, which is the final phase of wound healing process that eventually determines the strength and appearance of the healed tissue (Mantle *et al*., 2001).

Plant extracts have been used as a wound healing agents for long time (Wang *et al*., 2002). The use of traditional medicinal remedies and plants in the treatment of burns and wounds is viewed as an essential mode to improve healing processes and also to reduce the financial burden especially in the economically deprived societies of the developing world. Several plants and herbs have been used experimentally to treat skin disorders such as wound injuries in traditional medicine (Mahmood *et al*., 2009). There are a lot of studies have been done on natural herbs and plants for wound treatment. Most of the natural herbs and plants that have been studied contain active compounds like triterpenes, alkaloids, flavonoids and other biomolecules, that reported assisted in boosting and promoting the process of wound healing via influencing one or more of the phases of the healing process (Phillipson, 2001; Soma *et al*., 2012). It is reported that extracts of the medical plants and herbal as well as natural compounds involve in different and a few stages of wound healing which include coagulation, inflammation, fibroplasia, collagenation, epithelisation and wound contraction (Hemmati and Mohammadian, 2000).

In this study, three herbal extracts namely *Morinda citrifolia* fruits, *Melastoma malabathricum* and *Lawsonia inermis* leaves were investigated for their effectiveness in accelerating wound healing process. *M. citrifolia* is one of the most significant herbs that used in wound healing researches. All findings of the studies reported that *M. citrifolia* reduced the period of wound healing via reducing the period of inflammation phases, increasing the rate of cells proliferation and increase the level of epithelisation (Shivananda Nayak *et al*., 2007; Vijaykumar *et al*., 2008; Afa Palu *et al*., 2010). *M. malabathricum* is another important plant used in wound healing researches, especially in Malaysia. There are various studies done on *M. malabathricum* on the wound healing activities of this herb. A few studies recorded that *M. malabathricum* reduced the time of bleeding and repair of scar formation (Sulaiman *et al*., 2004; Sunilson *et al*., 2008; Manicam *et al*., 2010; Choudhury *et al*., 2011; Nurdiana and Marziana, 2013).

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L. inermis is also one of the significant herbs employed in traditional medicine for wound treatment. A study conducted on the effects of *L. inermis* on wound healing activities indicated that *L. inermis* reduced time of inflammatory phase of wound healing, increase epithelisation and scar formation (Sakarkar *et al*., 2004).

Problem statement

Recently, the necessity for the improvement of treatment of wound healing using natural herbs instead of using chemicals has been increased. This is attributed to the side effects of using chemical treatment, difficulties of preparing chemicals and the expenses. Although numerous studies have been conducted on wound healing using natural herbs including *M. citrifolia*, *M. malabathricum* and *L. inermis*, none of the studies investigated the effects of mixtures of these three herbs in accelerating wound healing process.

Hypothesis

It is hypothesised that mixtures of the three herbs will increase the activity of wound healing in rats.

Objectives

The aim of this study was to evaluate the effects of topical application of mixtures of *M, citrifolia, M. malabathricum*, and *L. inermis* on excision wound healing process in male Sprague Dawley rats.

The specific objectives of this study were:

1. to investigate the acute dermal toxicity of ethanolic extracts of *M. citrifolia* fruits, *M. malabathricum* leaves, and *L. inermis* leaves at doses of 2000 mg/kg and 5000 mg/kg body weight in female rats,

2. to investigate the sub-acute dermal toxicity of ethanolic extracts of *M. citrifolia* fruits, *M. malabathricum* leaves, and *L. inermis* leaves at doses of 500 mg/kg, 1000 mg/kg, and 2000 mg/kg body weight in male rats, and

3. to evaluate the effectiveness of mixtures of the herbal extracts at different concentrations for topical treatment of excision wounds via macroscopic, microscopic examination.

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