Faculty of Medicine and Health Sciences

Wound Healing Effects of *Litsea garciae* Extracts: *In vitro* Study on Fibroblast Cells

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Wound healing is a complex physiological process that involves regeneration of new cells and substitution of damaged cells after the onset of lesion. Wound healing involves the collaboration of many cell types and categorised into four overlapping main phases: haemostasis, inflammation, proliferation and remodelling. Although various wound healing treatments are currently available, the long-term usage of the medications has caused some adverse effects. To date, many traditional medicinal plants have been studied and widely used for various diseases due to their high efficacy, safety, and low cost. *Litsea garciae* (*L. garciae*) has many traditional medicinal uses and has been used by the indigenous people of Borneo Island to treat various diseases. However, its wound healing potential in skin wound therapy has not yet been investigated. Therefore, this study aimed to investigate whether the lipid and methanolic extracts of *Litsea garciae* could facilitate the process of *in vitro* wound healing and has a beneficial effect on the proliferation and migration of fibroblasts during *in vitro* wound healing. Total lipids were extracted using two different solvents: petroleum ether (PE) and Bligh and Dyer (BD). In addition, the crude methanolic extracts were prepared using 80% methanol. Firstly, the fatty acids composition of the lipid pulp and seed extracts of *L. garciae* were analysed using gas chromatography-mass spectrometry (GC-MS). The antimicrobial activities of the *L. garciae* lipid and methanolic pulp and seed extracts were evaluated through disk diffusion and broth microdilution assays. For the investigation of the potential wound healing properties of the *L. garciae* extracts, an *in vitro* study on the migration and chemotactic motility of the human dermal fibroblasts were conducted using wound scratch assay and transwell assay respectively. The effects of *L. garciae* extracts on the stimulation of the collagen type I, epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) expression levels were evaluated using

enzyme-linked immunosorbent assays (ELISA). This study showed that the prominent fatty acids in the *L. garciae* lipid seed extracts were lauric acid, palmitic acid, oleic acid, and linoleic acid. While for the *L. garciae* lipid pulp extracts, the predominant fatty acids were palmitic acid, stearic acid, oleic acid, and linoleic acid. All the *L. garciae* extracts exhibited mild antibacterial activities against the Gram-positive bacteria while Gram-negative bacteria exerted a weaker susceptibility towards the *L. garciae* extracts. The lipid pulp extracts were found to exhibit a stronger antimicrobial activity than the methanolic pulp extract while methanolic seed extract showed higher inhibition effect as compared to the lipid seed extracts. As for the wound healing property investigations, there were enhancement in the migration and chemotactic motility of fibroblasts treated with all the *L. garciae* pulp and seed extracts, but not with the methanolic seed extract. The expression levels of the collagen type I, EGF and VEGF by the wounded fibroblasts were ameliorated in the presence of the *L. garciae* extracts. These findings validated the ability of *L. garciae* extracts in promoting the wound healing process, which might be attributed to the presence of phytochemicals and fatty acids in the extracts. This study suggested that the *L. garciae* pulp and seed extracts might be a potent medication for skin wound healing.

Keywords: Wound healing, *Litsea garciae*, fibroblast, fatty acids, antimicrobial

Kesan-kesan Penyembuhan Luka bagi Ekstrak Litsea garciae: Kajian in vitro pada Sel Fibroblas

ABSTRAK

Penyembuhan luka merupakan proses fisiologi yang kompleks di mana ia melibatkan regenerasi sel-sel baru dan penggantian sel-sel yang rosak selepas kelukaan. Penyembuhan luka melibatkan kerjasama banyak jenis sel dan dikategorikan kepada empat tahap utama yang bertindih: hemostasis, inflamasi, proliferasi, dan pembentukan semula. Walaupun kini terdapat pelbagai cara bagi rawatan luka, tetapi penggunaan ubat-ubatan dalam jangka masa yang lama telah membawa kesan sampingan. Pada masa ini, banyak tumbuh-tumbuhan yang digunakan dalam perubatan tradisional telah dikaji dan digunakan secara meluas untuk merawat pelbagai penyakit disebabkan oleh keberkesanannya yang tinggi, keselamatan dan kos yang rendah. Litsea garciae (L. garciae) mempunyai banyak kegunaan dalam perubatan tradisional telah digunakan oleh bumiputera di Pulau Borneo untuk merawat pelbagai penyakit. Walau bagaimanapun, potensi tumbuhan ini dalam penyembuhan luka belum lagi dikaji. Oleh itu, kajian ini bertujuan untuk menyelidik sama ada ekstrak lipid dan metanol L. garciae dapat memudahkan proses penyembuhan luka dan mempunyai kesan yang bermanfaat terhadap proliferasi dan migrasi fibroblas dalam penyembuhan luka in vitro. Lipid diekstrak dengan menggunakan dua pelarut yang berbeza: eter petroleum (PE) dan "Bligh and Dyer" (BD). Selain itu, ekstrak metanol mentah disediakan dengan menggunakan 80% metanol. Pertamanya, komposisi asid lemak bagi ekstrak isi dan biji L. garciae yang diekstrak dengan PE dan kaedah BD telah dianalisa dengan menggunakan kromatografi gas-spektrometri jisim (GC-MS). Aktiviti antibakteria bagi ekstrak lemak dan metanolik L. garciae telah dinilai melalui ujian difusi cakera dan ujian mikrodilusi larutan. Bagi penyiasatan potensi ekstrak L. garciae atas sifat

penyembuhan luka, kajian in vitro pada migrasi dan motiliti kemotaktik bagi fibroblas kulit manusia telah dijalankan dengan menggunakan ujian goresan luka dan ujian "transwell" masing-masing. Kesan-kesan ekstrak L. garciae pada peningkatan tahap ekspresi kolagen jenis I, faktor pertumbuhan epidermis (EGF) dan faktor pertumbuhan endothelial vaskular (VEGF) telah dinilai melalui esei imunoserapan berkaitan enzim (ELISA). Hasil daripada penyelidikan ini mendapati bahawa asid lemak yang menonjol dalam ekstrak biji lipid L. garciae ialah asid laurik, asid palmitik, asid oleik dan asid linoleik. Manakala untuk ekstrak isi lipid L. garciae, asid lemak yang dominan ialah asid palmitik, asid stearik, asid oleik dan asid linoleik. Semua ekstrak L. garciae menunjukkan aktiviti antibakteria yang sederhana terhadap bakteria Gram-positif manakala bakteria Gram-negatif menunjukkan kerentanan yang lebih lemah terhadap ekstrak L. garciae. Ekstrak isi didapati mempunyai aktiviti antibakteria yang lebih kuat daripada ekstrak isi metanolik manakala ekstrak biji metanolik menunjukkan kesan perencatan yang lebih tinggi berbanding dengan ekstrak biji lipid. Bagi penyiasatan sifat penyembuhan luka, terdapat peningkatan dalam migrasi dan motiliti kemotaktik untuk fibroblas yang dirawat dengan semua ekstrak isi dan biji L. garciae, kecuali ekstrak biji metanolik. Tahap ekspresi kolagen jenis I, EGF dan VEGF yang dihasilkan oleh fibroblas telah meningkat apabila dirawat dengan ekstrak L. garciae. Dapatan kajian ini mengesahkan keupayaan ekstrak L. garciae dalam meningkatkan proses penyembuhan luka. Hal ini mungkin disebabkan oleh kewujudan fitokimia dan asid lemak dalam ekstrak tersebut. Kajian ini mencadangkan potensi ekstrak isi dan biji L. garciae sebagai ubat yang berkesan untuk penyembuhan luka kulit.

Kata kunci: *Penyembuhan luka, Litsea garciae, fibroblas, asid lemak, antibakteria*

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LIST OF ABBREVIATIONS

AAP	*Auricularia auricula-judae*
ABTS	2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid
AEAC	Ascorbic acid equivalent antioxidant capacity
ALA	α-linolenic acid
ANOVA	Analysis of Variance
BD	Bligh and Dyer
BDPE	Bligh and Dyer pulp extract
BDSE	Bligh and Dyer seed extract
bFGF	Basic fibroblast growth factor
BHT	Butylated hydroxytoluene
BSC	Biological safety cabinet
CH_3Cl	Chloroform
CHD	Coronary heart disease
CLSI	Clinical and Laboratory Standards Institute
CO_2	Carbon dioxide
CTGF	Connective tissue growth factor
DCF	*Digitaria ciliaris* flower
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPBS	Dulbecco's phosphate buffered saline
DPPH	2,2-Diphenyl-1-picryl-hydrazyl-hydrate
E. coli	*Escherichia coli*

ECM	Extracellular matrix
EFA	Essential fatty acids
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
Erk	Extracellular signal-regulated kinase
FabI	Enoyl-acyl carrier protein reductase
FAMEs	Fatty acid methyl esters
FBS	Foetal bovine serum
FD	Freeze-drying
FDA	Food and Drug Administration
FGF	Fibroblast growth factor
FRAP	Ferric reducing/antioxidant power
GC-MS	Gas chromatography-mass spectrometry
GML	Glycerol monolaurate
H_2O	Water
HCl	Hydrochloric acid
HDF	Human dermal fibroblasts
HO	Hyaluronidase
HRP	Horseradish peroxidase
HSD	Honestly Significant Different
IC_{50}	Half maximal inhibitory concentration
IL-1	Interleukin-1
KGF	Keratinocyte growth factor
L. garciae	*Litsea garciae*
LA	Linoleic acid

LCFAs	Long-chain fatty acids
LC-PUFA	Long chain polyunsaturated fatty acids
LC-SFA	Long-chain saturated fatty acids
LDL	Low-density lipoprotein
LNO	*Lucuma* nut oil
LO	Lipoxygenase
MAPK	Mitogen-activated protein kinase
MCFAs	Medium-chain fatty acids
ME	Methanolic
MeOH	Methanol
MEPE	Methanolic pulp extract
MESE	Methanolic seed extract
MHA	Mueller-Hinton agar
MHB	Mueller-Hinton broth
MIC	Minimum inhibitory concentration
MRSA	Methicillin-resistant *S. aureus*
MSC	Mesenchymal stem cells
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
MUFAs	Monounsaturated fatty acids
NaOH	Sodium hydroxide
NIST	National Institute of Standards and Technology
NO	Nitric oxide
NSAIDs	Nonsteroidal anti-inflammatory drugs
P. aeruginosa	*Pseudomonas aeruginosa*
PDGF	Platelet-derived growth factor

PE	Petroleum ether
PEPE	Petroleum ether pulp extract
PESE	Petroleum ether seed extract
PFA	Paraformaldehyde
PUFAs	Polyunsaturated fatty acids
RDFP	Red dragon fruit peel
S. aureus	*Staphylococcus aureus*
S. epidermidis	*Staphylococcus epidermidis*
SCFAs	Short-chain fatty acids
SD	Standard deviation
SFAs	Saturated fatty acids
SFM	Serum-free medium
SHSD	Superheated-steam drying
SPP	Saudi pomegranate peel
SPSS	Statistical Package for the Social Science
TFA	Trifluoroacetic acid
TGF	Transforming growth factor
TMB	3,3′,5,5′-Tetramethylbenzidine
TNF	Tumour necrosis factor
UNIMAS	Universiti Malaysia Sarawak
v/v	Volume/volume
VEGF	Vascular endothelial growth factor
VLCFAs	Very long chain fatty acids
XO	Xanthine oxidase

CHAPTER 1

INTRODUCTION

1.1 Background of Study

Skin is a protective barrier against various pathogens and when this barrier becomes compromised, the body responds by initiating a sequential repair process known as wound healing. Wound healing is a complex and highly regulated biological process to replace and reestablishes impaired tissues. The normal wound healing event can be disrupted by some co-morbidities such as diabetes, obesity and malnutrition (Anderson & Hamm, 2014; Han & Ceilley, 2017). This may result in chronic wounds which brings a burden to the patients and health care system due to the high costing of chronic wound treatment.

Although there are a lot of wound healing treatments and medication options already available for wound healing, there is still a necessity to research new efficacious medicine, especially using a medicinal plant. The medications commonly used in wound care include antibiotics and nonsteroidal anti-inflammatory drugs (NSAIDs). However, there are adverse effects attributed to the long-term usage of antibiotics and NSAIDs. The popularity of researching medicinal plants could be due to the perception that plants will cause minimal unwanted side effects and are less costly and more efficacious.

Litsea garciae (*L. garciae*) is known locally as engkala. Previous studies showed that the bark, leaf and fruit of *L. garciae* possessed antioxidant, antimicrobial, antifungal, anti-inflammatory, and anticancer properties. In addition, *L. garciae* also contained various phytochemical components such as alkaloids, butenolide, terpenes, flavonoids, amides, lignans, steroids and fatty acids. The indigenous people in Sarawak claimed that the leaves,