UNIVERSITI PUTRA MALAYSIA

EVALUATION OF DIABETIC WOUND HEALING PROPERTIES OF Moringa oleiferaLAM USING In vitroAND In vivoWOUND MODELS

ABUBAKAR MUHAMMAD AMALI

FPSK(p) 2015 14

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By

ABUBAKAR MUHAMMAD AMALI

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia, in Fulfillment of the Requirements for the Degree of Doctor of Philosophy

September 2015

DEDICATION

This thesis is dedicated to my late father, Alhaji Muhammad Amali. May Almighty Allah forgive him all his short comings and make Jannatul firdaus be his final abode amin.

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfillment of the requirements for the Degree of Doctor of Philosophy

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Chair: **Associate Professor Sharida Fakurazi, PhD**
Faculty: **Medicine and Health Sciences**

Diabetic wound is a common complication which affects significant number of people with diabetes. Its treatment is often very difficult which imposes burden and high cost on patients, family and society. Current treatments of diabetic wound are not sufficient enough with limited success in addition to non-affordability. *Moringa oleifera* Lam (*M. oleifera*) from the family *Moringaceae* (genus *Moringa*) commonly called drumstick or horseradish is a plant traditionally employed in the treatment of many ailments and has been scientifically proven to possess hepatoprotective, anti-inflammatory, antioxidant and hypoglycemic action in addition to other numerous activities.

The present study was undertaken to evaluate the potential of *M. oleifera* on wound healing in diabetic condition with a view to providing possible cost effective therapeutic alternative for treating diabetic wound topically. *In vitro* and *in vivo* wound models were utilized for our study.

The study initially demonstrated screening of crude extracts of methanol, ethanol and aqueous from *Moringa oleifera* leaves. Among these three solvent crude extracts, the methanolic crude extract was found to be the most active crude extract following the *in vitro* screening. The most active methanolic extract was then further subjected to bio-assay guided fractionation using hexane, dichloromethane, ethyl acetate, butanol and aqueous. The aqueous fraction was proven to be the most active fraction obtained from the results of *in vitro* screening and bio-guided assay fractionation.

The *in vitro* study included scratch test and proliferation assays using human dermal fibroblast cells (HDF), in which three different solvent crude extracts were screened and the most active methanolic crude extract was further subjected to differential bio graded assay fractionation. The most active aqueous fraction was finally obtained. HPLC, LC-MS/MS and UV spectroscopy were used for the identification and confirmation of bioactive compounds. Kaempferol and quercetin were identified in the crude methanolic extract while an active compound vicenin-2 was identified, confirmed and quantified in the bioactive aqueous fraction. Antioxidant and antibacterial assays were also conducted.

The *in vivo* study involved topical application of the formulated bioactive fraction using full thickness excision wound model in streptozotocin (STZ) and nicotinamide (NAD) induced diabetic rats. Healthy adult male Wistar rats weighing between 150-250g were used. Animals were grouped into six, consisting of six rats in each group (n=6): Two groups of normal and

diabetic controls, three groups of 0.5%, 1% and 2% w/w, aqueous fraction treated and one group of positive control that received 1% w/w silver sulfadiazine as standard drug. Treatments were applied topically in form of cream to the skin wounded area for 21 days. Biophysical, biochemical and histological parameters were evaluated. Proinflammatory cytokines analyses were performed using ELISA, Western blotting and immunohistochemistry techniques. Results were analyzed using SPSS version 20. Data were expressed as mean ± standard deviation, and results were selected from at least three independent experiments performed in triplicate. *P*-values of 0.05 were considered to be statistically significant.

The *in vitro* test results demonstrated that, crude methanolic extract and aqueous fraction of *M. oleifera* significantly stimulated proliferation and migration of HDF cells ($p<0.05$) at 24, 48 and 72 hours after treatment to close the artificially wounded area when compared to untreated control cells. The distance was measured and analyzed quantitatively at time interval of 0, 24, 48 and 72 hrs after the scratch. The MTT assay results showed that, aqueous fraction was relatively non-toxic and did not affect the cellular activity of HDF cells even at concentrations of 800 µg/mL after 72 hours. The aqueous fraction was tested and found to be effective in enhancing wound healing *in vitro* through proliferation and migration of human dermal fibroblast cells. In addition, antioxidant and antibacterial activities demonstrated by the bioactive aqueous fraction through radical scavenging and ferric reducing abilities as well inhibition of growth of *S. aureus*, *Ps. aeruginosa* and *E. coli* bacterial pathogens.

Following induction of diabetes by STZ-NAD in Wistar rats, hyperglycemia was maintained for 21 days and the reading of blood glucose level was more significant in diabetic groups compared to normal control group ($p < 0.05$). There was also some form of partial destruction of Islet of Langerhans and some normal islets were seen to be preserved even after the administration of a low dose of STZ and NAD which mimics the type-2 diabetes seen in humans.

The *in vivo* topical applications of various doses (0.5%, 1% and 2%) of bioactive aqueous fractions was found to be effective in enhancing diabetic wound healing through overall decreased wound size, improved wound contraction, enhanced tissue regeneration and granulation tissue, the decrease wound size in diabetic treated groups was more significant compared to untreated diabetic control group ($P < 0.05$) and there was also significant difference in contraction rate between diabetic treated groups compared to untreated diabetic control group ($P < 0.05$).

The topical application of aqueous fraction to the wound of diabetic animals caused down regulation of inflammatory mediators (TNF-α, IL1-β, IL-6, iNOS and COX-2) that was very significant in the diabetic treated groups as compared to non-treated diabetic control animals ($p<0.05$). The up regulation of VEGF protein in the diabetic treated groups was also found to be more significant ($P < 0.05$) compared to untreated diabetic control group that had less expression of VEGF.

Down regulation of inflammatory mediators and up regulation of VEGF with M. oleifera aqueous fraction facilitates overall wound healing and closure in diabetic condition. The bioactive compounds present in aqueous fraction have also been successfully identified and confirmed by HPLC and LC-MS/MS using standard vicenin-2 compound. These findings suggested that, topical administration of bioactive aqueous fraction of *M. oleifera* containing Vicenin-2 compound may accelerate wound healing in hyperglycemic condition. Therefore, may serve as a lead in drug discovery for diabetic wound healing.

Abstrak tesis yang dikemukakan kepada senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk Ijazah Doktor Falsafah

MENILAI SIFAT PENYEMBUHAN *Moringa oleifera* LAM TERHADAP LUKA DIABETIK MENGGUNAKAN MODEL LUKA *In Vitro* DAN *In Vivo*

Oleh

ABUBAKAR MUHAMMAD AMALI

September 2015

Pengerusi: Prof. Madya Sharida Fakurazi, PhD
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Rawatan selalunya sangat sukar dan membebankan pesakit, keluarga dan masyarakat dengan kos yang tinggi. Rawatan luka diabetik yang ada sekarang tidak mencukupi kerana kejayaan yang terhad ditambah oleh faktor ketidakmampuan. *Moringa oleifera* Lam (*M. oleifera*) daripada keluarga *Moringaceae* (genus *Moringa*) yang biasanya dipanggil kelor atau tumbuhan remunggai secara tradisi digunakan dalam rawatan pelbagai penyakit dan telah secara saintifiknya terbukti bahan aktif menghalang hati, antiradang, antioksidan dan tindakan hipoglisemik dan lain-lain.

Mula-mula kajian ini menunjukkan ujian ekstrak metanol, etanol dan air mentah daripada daun *Moringa oleifera.* Di antara tiga ekstrak pelarut mentah ini, ekstrak mentah etanol didapati sebagai ekstrak mentah paling aktif berdasarkan ujian *in vitro.* Ekstrak methanol mentah yang aktif ini kemudian menjalani pemeringkatan berpandu asai biologi menggunakan heksana, diklorometana, etil asetat, butanol dan air. Bahagian berair terbukti sebagai bahagian paling aktif yang didapatkan daripada hasil ujian *in vitro* dan pemeringkatan berpandu asai biologi.

Kajian *in vitro* termasuklah ujian calar dan pelbagai asai menggunakan sel-sel dermis fibroblas (SDF/HDF) manusia, yang tiga ekstrak pelarut diuji dan ekstrak paling aktif metanolik telah menjalani pemeringkatan asai gred biologi pembeza. Bahagian berair yang paling aktif akhirnya diperoleh. HPLC, LC-MS/MS dan spektroskopi UV digunakan bagi pengenalan dan pengesahan kompaun bioaktif. Kaempferol dan kuersetin telah dikenal pasti terdapat di dalam ekstrak mentah metanolik manakala kompaun aktif vicenin-2 telah dikenal pasti, disahkan dan didapati di dalam bahagian berair bioaktif. Pengesahan untuk antioksidan dan antibakteria juga dilakukan.

Kajian *in vivo* yang melibatkan aplikasi topikal bahagian bioaktif yang dilakukan menggunakan model eksisi luka penuh terhadap tikus diabetik yang diaruh oleh streptozotosin (STZ) dan nikotinamida (NAD). Tikus jantan Wistar dewasa yang sihat yang beratnya antara 150-250g telah digunakan. Haiwan ini dibahagiakan kepada enam kumpulan, yang mengandungi enam ekor tikus bagi setiap kumpulan (n=6): Dua kumpulan adalah kumpulan kawalan untuk normal dan menghidap diabetik, tiga kumpulan bagi 0.5%, 1% dan 2% w/w, dirawat dengan bahagian berair dan satu kumpulan kawalan positif yang menerima 1% w/w silver sulfadiazine sebagai dadah piawai. Rawatan dijalankan secara topikal dalam bentuk krim disapu pada kawasan yang luka selama 21 hari. Parameter biofizik, biokimia dan histologikal telah dinilai. Analisis pro keradangan sitokon telah

dilakukan menggunakan teknik ELISA, *Western blotting* dan imunohistokimia. Hasil kajian dianalisis menggunakan SPSS versi 20. Data dinyatakan dalam min ± sisihan piawai, dan hasil telah dipilih daripada sekurang-kurangnya tiga eksperimen berasingan yang dilakukan sebanyak tiga kali. Nilai-*P* adalah 0.05 dianggap signifikan secara statistik.

Hasil ujian *in vitro* menunjukkan, ekstrak mentah metanolik dan bahagian berair *M. oleifera* secara signifikan merangsang peningkatan dan perpindahan sel (SDF/HDF) ($p<0.05$) dalam tempoh 24, 48 dan 72 jam selepas rawatan dan menutup kawasan luka secara artifisal berbanding sel kawalan yang tidak mendapat rawatan. Jarak telah diukur dan dianalsisi secara kuantitatif pada selang masa 0, 24, 48 dan 72 jam selepas tercalar. Hasil asai MTT menunjukkan, bahagian berair berkeadaan tidak toksid secara relatifnya dan tidak memberi kesan pada aktiviti selular sel SDF/HDF walaupun pada tahap kepekatan 800 µg/mL selepas 72 jam. Bahagian berair telah diuji dan didapati berkesan dalam meningkatkan penyembuhan luka *in vitro* melalui peningkatan dan perpindahan sel dermis fibroblas manusia. Selain itu, aktiviti antioksidan dan antibakteria yang ditunjukkan oleh bahagian berair bioaktif melalui kebolehan hapus sisa radikal dan pengurangan ferik serta perencatan pertumbuhan patogen bakteria *S. aureus*, *Ps. aeruginosa* dan *E. Coli*.

Selepas tikus Wistar diaruh dengan diabetik menggunakan STZ-NAD, hiperglikemia dikekalkan selama 21 hari dan tahap glukos dalam darah meningkat dengan signifikan bagi kumpulan diabetik berbanding kumpulan kawalan yang normal ($p < 0.05$). Selain itu berlaku sedikit kerosakan terhadap kelompok Langerhans dan sebahagian kelompok kelihatan terpelihara walaupun selepas dos rendah STZ dan NAD diberikan bagi menyerupai diabetik jenis-2 pada manusia.

Aplikasi topikal in vivo pelbagai dos (0.5%, 1% dan 2%) bahagian berair bioaktif didapati aktif dalam meningkatkan penyembuhan luka diabetik dengan secara keseluruhan mengurangkan saiz luka, pengecutan luka yang bertambah elok, peningkatan pertumbuhan semula tisu dan tisu granulasi, penurunan saiz luka pada kumpulan rawatan diabetik adalah lebih signifikan berbanding kumpulan diabetik tidak dirawat ($P < 0.05$) dan juga terdapat perbezaan yang signifikan dalam kadar pengecutan antara kumpulan rawatan diabetik berbanding kumpulan kawalan tidak dirawat ($P < 0.05$).

Aplikasi topikal bahagian berair terhadap luka haiwan diabetik menyebabkan pengawalaturan menurun pengantara keradangan (TNF-α, IL1-β, IL-6, iNOS dan COX-2) yang didapati sangat signifikan dalam kumpulan kawalan diabetik berbanding kumpulan haiwan kawalan diabetik yang tidak dirawat ($p<0.05$). Pengawalaturan menaik bagi protein VEGF dalam kumpulan kawalan rawatan juga didapati lebih signifikan ($P < 0.05$) berbanding kumpulan kawalan diabetik yang tidak dirawat yang mengandungi kurang VEGF.

Kesemua faktor ini menyumbang kepada keseluruhan penyembuhan dan penutupan luka pesakit diabetik. Kompaun bioaktif yang terdapat dalam bahagian berair juga Berjaya dikenal pasti dan disahkan oleh HPLC dan LC-MS/MS menggunakan piawai kompaun vicenin-2. Dapatan ini menunjukkan, aplikasi topikal bahagian berair bioaktif *M. oleifera* yang mengandungi kompaun Vicenin-2 dapat mempercepatkan penyembuhan luka dalam keadaan hiperglikemik. Oleh itu, ia membawa kepada penemuan ubat untuk menyembuhkan luka diabetik.

ACKNOWLEDGEMENTS

I wish to begin by thanking Almighty Allah, the Most Gracious and Most Merciful, Who bestow on me the blessings and wisdom to live up to the stage of completing this research project despite many challenges, I pray for His continuous guidance and blessings till the end of my last breath, amin. I will like to express my appreciation to all those who supported me during these years of struggle, in particular: Associate Prof Dr Sharida Binti Fakurazi, my supervisor and advisor, for fine tuning me to scientific research and experimental Pharmacology and letting me share your great knowledge and experience. Your warm and generous support, endless enthusiasm, great concern have been invaluable.

My appreciation goes to my co-supervisors, Associate Prof, Dr Faridah Abas, for excellent advice and support, and for giving me the freedom to combine my Pharmacological knowledge with Phytochemistry research. To Dr Zalinah Ahmad, I remain grateful for sharing exciting sources of inspiration and knowledge in your field of specialty. Dr Cheah Pike See, for excellent advice, professional and skillful technical assistance especially in areas related to histhopathology and immunohistochemistry.

Many thanks to Dr Palanisamy Arulselvan for helping me straighten out my question marks in the field of Proteomics and Scientific writing. I wish to also thank all Staff of Laboratory of Vaccine and Immunotherapeutics, for the enormous help, and for creating such a positive and enabling atmosphere in the laboratory.

My colleagues and research mates; Abdulmalik, Aminu, Aimi, Shafinaz, Tina, Karthy, Atiqah, Sani, Aminu, Ashiru, Rabiu, Sani, UDUS Scholars and the Naija community members in UPM for the interactions, sharing of ideas and joyful moments, it's been a pleasure being part of you all. To all my friends and colleagues back home in Nigeria, too numerous to mention, for many years of friendship, I cherish all of you for the wonderful moments we had together.

Many thanks to my brothers and sisters back home in Nigeria, I salute all the encouragements and prayers offered me at all times for the great success, May Allah bless you all, amin.

To my departed father, of blessed memory, May Allah forgive him all his short comings and bless him with jannatul firdaus amin. To my living mother, who has been supportive all the way and gave me all the courage to pursue my interests and always being there for me during challenging moments of my life with non-stop prayers and admonishments, I owe you a debt that can never be paid back, all I will say therefore, May Almighty Allah reward you abundantly and continue to shower His blessings on You here in the world and the hereafter, amin.

To, my dear wife and partner, Hajiya Fatimah, Our children, Aisha and Muhammad, Your great care, invaluable support, patience, love and understanding will remain in my memory for ever and ever. I love you all!

I sincerely thank you all.

This thesis was submitted to the Senate of Universiti Putra Malaysia and has been accepted as fulfillment of the requirement for the degree of Doctor of Philosophy. The members of the Supervisory Committee were as follows:

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABREVIATIONS

ADA	American diabetic association
AGE	Advanced glycation end products
BSA	Bovine serum albumin
BMMSC	Bone marrow derived mesenchymal cells
COX-2	Cyclooxygenase -2
DAB	3,3-diaminobenzene
DMEM	Dulbecco's modified eagle's medium
DFU	Diabetic foot ulcer
ECM	Extracellular matrix;
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor
ELISA	Enzyme-linked immunosorbent assay
EPC	Endothelial progenitor cell
FBS	Fetal bovine serum
FCS	Fetal calf serum
FGF	Fibroblast growth factor
GAG	Glycosaminoglycan
GSH	Reduced glutathione
H & E	Hematoxylin and Eosin
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IGF	Insulin-like growth factor
Il-1	Interleukin
IDF	International diabetic federation
i.p	Intraperitoneal
IC_{50}	Inhibitory concentration
IL-1β	Interleukin 1-beta
IL-6	Interleukin six
KGF	Keratinocyte growth factor
LCMS/MS	Liquid Chromatography-Mass Spectrometry

MDA	Malaysian diabetic association
MIC	Minimum inhibitory concentration
MMP	Matrix metalloproteinase
min	Minute
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
NAD.	Nicotinamide adenine dinucleotide
GF	Growth factor
NO	Nitric oxide
iNOS	Inducible nitric oxide synthase
PBS	Phosphate buffer saline
PDGF	Platelet-derived growth factor
PMN	Polymorphonuclear neutrophil
PVD	Peripheral vascular disease
PVDF	Polyvinylidene difluoride
RAGE	Receptors for Advanced Glycation End Products
ROS	Reactive oxygen specie
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
STZ	Streptozotocin
TEMED	Tetramethylethylenediamine
TGF β -R	Transforming Growth Factor β Receptor
TGF-β	Transforming Growth Factor β
TIMP	Tissue inhibitor of metalloproteinase
TMB	Tetramethylbenzidine
TNF α	Tumour Necrosis Factor α
UV/VIS	Ultraviolet visible
VEGF	Vascular endothelial growth factor
WHO	World health organization
w/w	Weight/weight
µm	Micrometer
%	Percentage
^{o}C	Degree celcius

CHAPTER 1

INTRODUCTION

1.1 Background

The Wound Healing Society defines wounds as physical injuries which result in an opening or break of the skin that disturb normal skin anatomy and function (Strodtbeck, 2001). It results in the loss of epithelium with or without loss of underlying connective tissue (Nagori and Solanki, 2011). Wound healing processes occur in a well-orchestrated combination of multifaceted biological and molecular events of cell migration, cell proliferation and extracellular matrix (ECM) deposition (Fulzele *et al*., 2002).

As the world is facing epidemic of type 2 diabetes and an increasing incidence of type 1 diabetes (Wild *et al*., 2004; Gale, 2002; Boulton *et al*; 2005), the International Diabetes Federation (IDF) focused on global burden of diabetic foot disease as a global concern. People with diabetes have a 12– 25% lifetime risk of developing foot ulcer (Singh *et al*., 2005; Falanga, 2005). High prevalence rates of diabetes in many countries of the world make foot ulcers a major and increasing public-health problem. Foot ulcers are known to cause substantial morbidity, impair quality of life, endangering high treatment costs (about US$17 500– 27 987 / UK£9533– 15 246) (Falanga, 2005). Since the lifetime risk of a person with diabetes developing a foot ulcer could be as high as 25%, and emergence of contributory pathogenic factors such as neuropathy and vascular disease that are present in more than 10% of people at the time of diagnosis of type 2 diabetes, then the burden of diabetic foot disease is set to increase in future (Group, 1998; Boulton *et al*., 2005). Even the first year of diagnosis of diabetes could be a period of danger for foot ulcers and amputations (New *et al*; 2000; Boulton *et al*., 2005).

Persistent hyperglycemia may negatively affect wound healing and immune system of the body (Brem *et al*., 2007). In diabetic patients, cell proliferation is often impaired, likely to undergo apoptosis, impairment of blood vessel growth and decreased deposition of collagen at the wound site. These factors contribute significantly to prolongation of injury thereby slowing the process of wound healing in diabetic condition.

Plants and chemical entities derived from plants have been identified and formulated for treatment and management of wounds. More of such herbal products are being investigated to date. Some of the plants that have been tested for diabetic wound healing include *Acalypha langiana* (Perez and Vargas, 2006), *Radix astragalis and Radix Rehmannia* (Lau *et al*., 2009), *Rosmanis officinalis* L (Abu-Al-Basal., 2010), *Curcuma longa* (Sidhu *et al*., 1999), *Sparassia crispa* (Kwon *et al*., 2009), *Hylocereus undatus* (Perez *et al*., 2005), *Momordica charantia* (Teoh *et al*., 2009), *Lithospermum erythrorhison* (Fujita *et al*., 2003), *Aloe vera* (Atiba *et al*., 2011).

Natural products have been widely used as source of therapeutic agents for the management of human diseases following their safety, efficacy and lesser side effects. The process of wound healing has been shown to be promoted by several natural products (Song and Salcido, 2011). Plants contain different bioactive principles such as alkaloids, flavonoids, tannins, and steroids. These agents may influence one or more phases of wound healing (Ponrasu and Suguna, 2012). Evaluation of various plant products according to their traditional uses and medicinal value based on their therapeutic efficacy leads to the discovery of newer and cost effective drugs for treating various ailments. This form the basis for our study to develop new agent from *M. oleifera* that may be useful in facilitating wound healing in hyperglycemic condition.

1.2 Statement of Problem

The underlying causes of risk factors leading to the onset of diabetic foot ulcers which include peripheral neuropathy (lack of sensation in poorly vascularized lower extremities) and can be motor, sensory and autonomic. Dry, stiff skin can crack easily and causes splits that can lead to infection, resulting in cellulitis or ulcerations which potentially leads to ultimate loss of the lower limb. (Tanenberg and Donofrio, 2008). Other factors such as environmental factors, peripheral vascular disease, a compromised immune system and poor metabolic control, in addition to social influences such as emotional, psychological and behavioural problems (Lyons, 2008) are all contributing risks that leads to diabetic ulcer.

One of the complications of diabetes is foot ulcer, known to be the main cause of prolonged hospital stays in developing countries, and are also known to cause substantial morbidity, impair quality of life and engender high treatment costs. The annual treatment cost of diabetic ulcer per patient stood at about US$17 500– 27 987 / UK£9533– 15 246 (Falang, 2005; Martin *et al*; (2009).

Despite holistic approach to treatment of diabetic foot ulcer (DFU) which involve tight glucose control and meticulous wound care, the prognosis is often quite poor. This results to amputation and physical disability that can occur even in first year of diagnosis of diabetes (New *et al*; 2000; Boulton *et al*., 2005). The lifetime risk of a person with diabetes developing DFU could be as high as 25%, and with the emergence of contributory pathogenic factors such as peripheral neuropathy and vascular disease, the burden of DFU is set to increase in the future as reported by the United kingdom prospective diabetic study (Group, 1998; Boulton *et al*., 2005). Developing countries in Africa and Asia are more affected by DFU due to high prevalence of type 2 diabetes. For example it was estimated that, 15% of all deaths in South East Asia were attributable to diabetes (IDF, 2011). In Malaysia for example, a 2 year retrospective study (2003-2005) showed that, out of 203 amputated patients at University Sains Malaysia Hospital, 134 (66%) were diabetic related amputations (Yusof *et al*; 2007).

1.3 Justification of the study

The future of diabetic wound treatment lies in research and development of effective agents that facilitates wound healing. Therefore, researchers, medical practitioners,

manufacturers of medicines and most importantly, diabetic patients, would all want to see a breakthrough in this area. The basic understanding of pathogenesis of diabetic wounds and its impaired healing may pave way for the development of novel and cost effective agents that enhances timely healing and closure of wound.

The present study may serve as a lead in discovering agent that promotes wound healing in diabetes. This may help reduce cost of hospitalization and save patients from amputations arising from complications. The study may also lead to better understanding of influence of natural products on chronic wound which forms a platform for further studies that open up possibility of finding alternative therapy for diabetic wound.

It is in the hope of improving these outcomes; we conduct an investigation into bioactive compounds from *Moringa oleifera* which may provide treatment alternatives for diabetic wound and contribute to an increased database of knowledge of phytomedicines in health and diseases.

1.4 Research hypothesis

The hypothesis to be tested in this study is that, topical application of *Moringa oleifera* enhances wound healing in hyperglycemic condition using *in vitro* and *in vivo* wound models.

1.5 Objectives of the study

1.5.1 General objective

The objective of the present study is to evaluate the wound healing efficacy of topical administration of *Moringa oleifera* in diabetes using *in vitro* and *in vivo* experimental wound models.

1.5.2 Specific objectives

1) To determine the most active crude extract and fraction of *M. oleifera* using *in vitro* bio-guided assay fractionation.
2) To identify and confirm bioactive compounds present in active crude extract and fraction using HPLC-DAD and LC-MS/MS analysis.
3) To evaluate the antibacterial activities of bioactive fraction of *M. oleifera*.
4) To evaluate the antioxidant activities of bioactive fraction of *M. oleifera*.
5) To evaluate wound healing efficacy of bioactive fraction of *M. oleifera* topically in hyperglycemic induced animal model.
6) To examine and evaluate histological changes of wound tissue samples in rats, following treatment with bioactive fraction of *M. oleifera.*
7) To evaluate expression levels of selected pro-inflammatory cytokines via analysis of wound tissue samples using ELISA, Western blotting and immunohistochemistry techniques.

1.6 Scope and relevance

There is controversy over how to treat poorly healing wounds like diabetic wounds depending on the stage of the wound, topical agents could be beneficial as supported by some evidences like the use of topical antibiotics and a state-of-the-art topical dressing (Sweitzer *et al*., 2006).

However, the management of diabetic foot is complex, requiring a multifactorial approach and therefore, use of topical application alone may have its own limitations because it is not effective for highly exuding wounds; rapidly absorbs fluid, loses its rheological characteristics, and becomes mobile as it remains on wounds for longer periods of time (Boateng *et al*., 2008). In these situations topical application has to be considered as an adjunctive therapy to systemic therapy. Although topical therapy is important, it is often insufficient and therefore DFU treatment requires additional wound care.

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