WOUND HEALING ACTIVITIES OF *ORTOSIPHON STAMINEUS* LEAVES AND PROTEIN EXTRACTS ON HUMAN SKIN FIBROBLAST IN DIABETIC MICROENVIRONMENT

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DEDICATION

It is my warmest regard and genuine gratefulness that I dedicate this work toThe Almighty, for always being at the centre of my life and for giving me this opportunity to seek and gain wisdom and knowledge. I also dedicate it to my parents for their limitless support, sacrifices and love to make my life in and out of universitybetter and for who I am today. A special dedication to my grandparents, siblings, aunties, uncles, nieces and nephews, it is because of them, I could face the world with a smile, their emotional support and guidance in helping me finish my task and reminding me to always take one step at a time. Not forgetting my friends, their endless support and trust for all that I do, ensuring me that I always do my best, motivates me to always be and do better.

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ABSTRACT

Orthosiphon stamineus, also known as cat whiskers, is a well-known plant in herbal remedies and traditional medicine. The ethanolic leaves and transketolase protein extracts of the plant may be a possible diabetic wound healing. It has previously been shown that this plant extract has potential antihyperglycemic and wound healing effects in *in vivo* on diabetic induced rats under normal and hyperglycemic conditions. However, the effects of the *O. stamineus* leave extracts on human skin fibroblast (HSF 1184) cells under hyperglycemic microenvironment and protein extracts on HSF 1184 cells under normal and hyperglycaemic microenvironments are still unknown. The aim of this research is to study the cell viability and wound healing activity of the *O. stamineus* ethanolic and protein extract on HSF 1184 cells under both microenvironments. The cytotoxicity and migration assay were conducted in a various selected range of ethanolic and protein extract concentrations against HSF 1184 cells using MTT assay and wound healing assay. The cytotoxicity assay was evaluated after 72 hours of treatment under different concentrations of *O. stamineus* ethanolic (7.81, 15.63, 31.25, 62.5, 125, 250, 500 and 1000 μg/mL) and protein extracts (1.25, 2.5, 5, 10 and 20 ng/mL) under both microenvironments. The results show that the highest cell proliferation activity at the concentration of *O. stamineus* ethanolic extract (7.81 μg/mL) and protein extract (1.25 ng/mL), respectively. Conversely, the highest concentration of *O. stamineus* ethanolic(1000 μg/mL) and protein extract (20 ng/mL) resulted in the lower cell proliferation activity under both microenvironments respectively. However, the *O. stamineus* ethanolic and protein extracts showed low cytotoxicity effects against the HSF 1184 cells under both microenvironments. The IC50 value obtained for the *O. stamineus* ethanolic extract under normal microenvironment was 151.36 μg/mL and under hyperglycemic microenvironment was 141.26 μg/mL respectively. Whereas, no IC50 values were obtained from the *O. stamineus* protein extracts. The wound healing assaywas determined based on the accelerated cell migration activity at 18, 20, 22 and 24 hours of incubation with various concentrations of *O. stamineus* ethanolic (40, 80 and 160 μg/mL) and protein extracts (5, 10 and 20 ng/mL) under both microenvironments. Based on the results for the *in vitro* wound healing assay of *O. stamineus* ethanolic extracts, control has shown to exhibit higher cell migrating activity at 86.99% compared to the remaining concentration under normal microenvironment. On the other hand, *O. stamineus* ethanolic extracts at the concentration of 40 μg/mL displayed rapid wound closure activity at 89.17% compared to control under the hyperglycemic microenvironment. Nevertheless, *in vitro* wound healing assay of *O. stamineus* protein extracts, treatment with 5 ng/mL has revealed a remarkable wound closure rate at 99.79% when compared to the control under normal microenvironment. While *O. stamineus* protein extracts at the concentration of 10 ng/mL showed enhanced wound closure effect at 99.83% when compared to control under hyperglycemic microenvironment. Collectively, these results suggested that *O. stamineus* ethanolic and protein extracts have potential *in vitro* in the acceleration of cell migration whichinfluences the traditional use of *O. stamineus* extracts in wound healing activity on HSF 1184 cells.

ABSTRAK

Orthosiphon stamineus, atau misai kucing, merupakan sejenis tumbuhan yang terkenal dalam perubatan herba dan tradisional. Ekstrak etanol daun dan ekstrak protein transketolase tumbuhan ini dipercayai dapat menyembuhkan luka diabetes. Kajian terdahulu telah menunjukkan bahawa ekstrak tumbuhan ini mempunyai kesan antihiperglikemik dan berpotensi terhadap penyembuhan luka *in vivo* pada tikus teraruh diabetes dalam keadaan normal dan hiperglikemik. Walau bagaimanapun, kesan ekstrak daun *O. stamineus* terhadap sel fibroblast kulit manusia (HSF 1184) dalam persekitaran mikro hiperglikemik serta kesan ekstrak protein terhadap sel HSF 1184 dalam persekitaran mikro normal dan persekitaran mikro hiperglikemik masih belum diketahui. Tujuan penyelidikan ini adalah untuk mengkaji daya maju sel dan aktiviti penyembuhan luka oleh ekstrak etanol dan ekstrak protein *O. stamineus* terhadap sel HSF 1184 dalam persekitaran mikro normal dan persekitaran mikro hiperglikemik. Ujian sitotoksik dan migrasi sel terhadap HSF 1184 dijalankan dengan kepekatan ekstrak etanol dan protein yang berbeza, menggunakan asai MTT dan asai penyembuhan luka. Ujian sitotoksik dinilai selepas 72 jam rawatan dijalankan dengan kepekatan berbeza ekstrak etanol O. *stamineus* (7.81, 15.63, 31.25, 62.5, 125, 250, 500 dan 1000 µg/mL) serta ekstrak protein (1.25, 2.5, 5, 10 dan 20 ng/mL) dalam persekitaran mikro normal dan hiperglikemik. Hasil ujian mendapati bahawa aktiviti proliferasi sel tertinggi ditunjukkan oleh kepekatan ekstrak etanol *O. stamineus* 7.81 µg/mL dan ekstrak protein 1.25 ng/mL. Namun begitu, kepekatan tertinggi ekstrak etanol *O. stamineus* (1000 µg/mL) dan ekstrak protein (20 ng/mL), telah menurunkan aktiviti proliferasi sel dalam kedua-dua persekitaran mikro. Ekstrak etanol dan protein *O. stamineus* juga telah menunjukkan kesan sitotoksik yang rendah terhadap sel HSF 1184 dalam kedua-dua persekitaran mikro. Nilai IC50 yang diperoleh untuk ekstrak etanol *O. stamineus* dalam persekitaran mikro normal adalah 151.36 µg/mL manakala dalam persekitaran mikro hiperglikemik adalah 141.26 µg/mL. Tiada nilai IC50 yang diperoleh daripada ekstrak protein *O. stamineus.* Sementara itu, ujian penyembuhan luka telah dijalankan melalui penentuan aktiviti migrasi sel yang dipercepatkan pada inkubasi 18, 20, 22 dan 24 jam dengan menggunakan kepekatan berbeza ekstrak etanol *O. stamineus* (40, 80 dan 160 µg/mL) dan ekstrak protein (5, 10 dan 20 ng/mL) dalam kedua-dua persekitaran mikro. Berdasarkan asai penyembuhan luka *in vitro* oleh ekstrak etanol *O. stamineus*, kawalan telah menunjukkan aktiviti migrasi sel yang lebih tinggi pada 86.99% berbanding kepekatan lain dalam persekitaran mikro normal. Ekstrak etanol *O. stamineus* dengan kepekatan 40 µg/mL telah menunjukkan aktiviti penutupan luka yang cepat pada 89.17% berbanding kawalan dalam persekitaran mikro hiperglikemik. Ekstrak protein *O. stamineus* dengan kepekatan 5 ng/mL telah menunjukkan kadar penutupan luka yang ketara pada 99.79% berbanding kawalan dalam persekitaran mikro normal, manakala ekstrak protein dengan kepekatan 10 ng/mL pula telah menunjukkan peningkatan kesan penutupan luka pada 99.83% berbanding kawalan dalam persekitaran mikro hiperglikemik. Secara keseluruhannya, dapatan ini menunjukkan bahawa ekstrak etanol dan ekstrak protein *O. stamineus* mempunyai potensi *in vitro* dalam mempercepatkan migrasi sel yang mempengaruhi penggunaan tradisional ekstrak *O. stamineus* dalam aktiviti penyembuhan luka terhadap sel HSF 1184.

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LIST OF ABBREVIATIONS

ABs	-	Absorbance
AGEs	-	Advanced End Products of Glycation
DAG	-	Diacylglycerol
DHT	-	Dihydrotestosterone
DMEM	-	Dulbecco's Modified Eagle Medium
DMSO	-	Dimethyl Sulfoxide
DNA	-	Deoxyribonucleic Acid
ECM	-	Extracellular Matrix
EDTA	-	Disodium Ethylenediaminetetraacetic Acid
EGF	-	Epidermal Growth Factor
EPCs	-	Endothelial Progenitor Cells
FBS	-	Fetal Bovine Serum
FGF	-	Fibroblast Growth Factor
HCI	-	Hydrochloric Acid
HLA	-	Human Leukocyte Antigen
HPLC	-	High-Performance Liquid Chromatography
IBD	-	Institute Bioproduct Development
IC_{50}	-	Half-maximal inhibitory concentration
ICA	-	Islet Cell Autoantibodies
MMPs	-	Matrix Metalloproteinase
MTT	-	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
PBS	-	Phosphate Buffer Saline
PDGF	-	Platelet-Derived Growth Factor
RPM	-	Revolutions Per Minute
SHRSP	-	Stroke-Prone Spontaneously Hypertensive Rats

SPSS	-	Statistical Package for Social Sciences
SSIs	-	Surgical Site Infections
STD	-	Standard Deviation
STZ	-	Streptozotocin
T2DM	-	Type 2 Diabetes Mellitus
TGF	-	Transforming Growth Factor
TGF-β	-	Transforming Growth Factor Beta Receptor
TIDM	-	Type I Diabetes Mellitus
TKT	-	Transketolase
TPP	-	Thiamine-Diphosphate
USA	-	United States of America
UTM	-	Universiti Teknologi Malaysia
WHO	-	World Health Organisation

LIST OF SYMBOLS

β	-	Beta
α		Alpha
μ	-	Micro
ℓ	-	Litre
%	-	Percent
μg	-	Microgram
µm		Micrometre
$m\ell$	-	Millilitre
℃	-	Degree Celsius
◦	-	Degree
g	-	Gram
mg	-	Milligram
ng	-	Nanogram
M	-	Molar
mM	-	Millimolar
cm	-	Centimetre
mm	-	Millimetre
(v/v)	-	Volume over volume
>	-	Greater than
<	-	Lesser than

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CHAPTER 1

INTRODUCTION

1.1 Problem Background

Diabetes mellitus is defined as insulin resistance because of pancreatic β-cell dysfunction due to unsettled hyperglycemia (Hameed *et al.*, 2015). The risk of this disease is enormous due to the shift in food intake and lifestyle, the aging of the urbanization and population in the form of a genetically influenced environment (Hameed *et al.*, 2015). The first report on diabetes was recorded in an Egyptian manuscript dated back to approximately 1500 B.C. (Alam *et al.*, 2017). The disease is characterized by symptomatic with chronic hyperglycaemia (i.e. high blood glucose level) due to cellular defects in insulin action, insulin secretion or both (Gupta *et al.*, 2015; Myers and Zonszein, 2004). The disruption of the insulin metabolism, which plays a crucial role as an anabolic hormone, resulting in metabolic abnormalities in lipids, proteins, and carbohydrates (Vargas, Joy and Sepulveda, 2018; Wilcox, 2005). Specifically, there are four classes of diabetes mellitus, regulated based on its clinical presentation and aetiology: Type I Diabetes Mellitus (TIDM), Type 2 Diabetes Mellitus (T2DM), gestational diabetes, and other specialized types (Piero *et al.*, 2015).

Wounds are any injury or damage to the internal structure and functions due to the extreme abrasion of organs like skin (Okur *et al.*, 2020). Wound healing is a natural process in which damaged tissue heals and restores to its previous state (Krafts, 2010; Velnar *et al.*, 2009). Wound healing is associated with a cascade of biomolecular mechanisms and complex cellular processes involving in repairing tissue injuries (Krafts, 2010; Velnar *et al.*, 2009). Overall, a wound healing cascade is achieved in three stages: inflammation, proliferation, and maturation (Sinno and Prakash, 2013).

The healing process starts with the initiation of the inflammatory phase, which is characterized by inflammation and hemostasis (Jameson and Havran, 2007). Next, the progressive development of normal wound healing comprises the integrated interaction of extracellular matrix components, proteases, blood cells, growth factors, and proteins (Tan *et al.*, 2019). The propelling mechanism responsible for increased mortality and morbidity of diabetes is related to vascular problems as well as to the inability of wound healing mechanisms in the diabetic state (Oguntibeju, 2019). As a result, wound healing is significantly delayed in diabetes and chronic wounds are frequent to occur (Okonkwo and Dipietro, 2017).

Chronic wounds are often among the most debilitating conditions in diabetic patients due to compromised immune response and subsequent high microbial burden, which often contribute to amputation, particularly of the lower limbs (Pereira *et al.*, 2017; Barshes *et al.*, 2013). It is estimated that a patient with diabetes has a 15-25% probability of acquiring a chronic wound (Gianino, Miller and Gilmore, 2018). Chronic wounds do not cure spontaneously, and some degree of clinical care is still required (Pereira *et al.*, 2017).

Therefore, effective clinical interventions are undertaken after the diagnosis of wound chronicity (Pereira *et al.*, 2017). Debridement of infected tissues, the use of extensive dressings and the application of a vast and sometimes limited variety of antibiotics are the most widely used treatment for diabetic chronic wounds (Pereira *et al.*, 2017; Lipsky *et al.*, 2016). Nevertheless, recovery rates are also far from being optimal, with fewer than 50% of patients responding significantly to this treatment. However, amputation and hospitalization at times are still needed, although the results achieved are insufficient for those patients (Pereira *et al.*, 2017; Barshes *et al.*, 2013; Forlee, 2011).

Though different prescription formulations are available, new medicinal options with much less adverse reactions, lower costs and reduced recovery time are highly demanded for clinical care (Lordani *et al.*, 2018). Various efforts have been made to improve wound healing in diabetics, but there are currently only a few successful medicinal treatments available (Tan *et al.*, 2019). Among other factors,

there seems to be a growing increase in the use of medicinal plants since they develop lower side effects and are inexpensive than the conventional drugs found over the counter (Okur *et al.*, 2020; Tan *et al.*, 2019; Duque *et al.*, 2016). Medicinal plants are probably one of the many methods that have received research attention globally (Oguntibeju, 2019). Until now, many traditional practitioners use herbs to make juices and remedies for diabetes, and such treatments are still practicing in Malaysia (Samuel *et al.*, 2010). Hence, this leads to the purpose of this study in using *Orthosiphon stamineus* (*O. stamineus*) as a potential medicinal plant to study the diabetic wound healing activity *in vitro*.

O. stamineus is a common traditional herbal plant typically referred to by the locals as *Misai Kucing;* in the Malay language, that means cat whiskers (Singh *et al.*, 2015). This plant is distributed extensively in Southeast Asia and other tropical regions (Basheer and Majid, 2010). Scientists and researchers have placed more focus on this plant to analyze its medicinal properties, especially concerning the pharmacological uses (Almatar *et al.*, 2013). Along with the medicinal properties found in *O. stamineus*, the proteins present in the plant may also act as an antihyperglycaemic agent. The study of protein population in a cell, tissue or subcellular compartment is known as proteomics (Shahzad *et al.*, 2016).

A proteome is a comprehensive collection of proteins that are expressed by a genome of an organism (Shahzad *et al.*, 2016). Among these proteomes, transketolase (TKT) is a protein found in the leaves of the plant where it is considered to have a role in lowering the blood sugar level *via* the human body's non-pancreatic mechanism (Ng, Rahmat, and Shamsir, 2018). Transketolase of *O. stamineus* can be a potential therapeutic agent to prevent diabetes and thus aid in diabetic wound healing as well (Chua and Abdullah, 2019).

1.2 Problem Statement

Diabetes mellitus is one of the significant challenges in global public health, with the dramatic increase in cases observed over the past two decades (Yazdanpanah, 2015). WHO reported that 347 people worldwide were diagnosed with diabetes in 2013, and the number is expected to double by 2030, and this can lead to an increase in treatment costs due to severe complications (Alqathama *et al.*, 2020). Therefore, efficiency in treating a chronic diabetic wound is crucial to reduce the cost of treatment. The consumption of commercially available drugs has been reported to associate with several side effects, such as liver and kidney disease (Farzaei *et al.*, 2017). The condition worsened when they were consumed for a long term (Kalsi *et al.*, 2015).

As several side effects have been reported, further research is necessary to discover safer treatments (Farzaei *et al.*, 2017). Some studies have reported that the consumption of herbal medicine can treat diabetic wounds (Okur *et al.*, 2020; Tan *et al.*, 2019; Choudhury *et al.*, 2018). One of the major therapeutic approaches used to treat diabetes mellitus is by the consumption of concoction several medicinal plants (i.e. their extracts) (Farzaei *et al.*, 2015). However, the scientific information and evidence on the bioactivity of the herbs in treating diabetes are still lacking, especially for the bioactivity of the *O. stamineus* plant in improving diabetic wound healing which will be used in this study (Choudhury *et al.*, 2018).

In this study, the extract and protein of the *O. stamineus* plant will be tested on HSF 1184 cells in a hyperglycaemic microenvironment. The effects of *O. stamineus* on diabetic wounds have been studied previously *in vivo* and *in vitro* in a normal microenvironment (Lokman *et al.*, 2019). However, to the best of our knowledge, *O. stamineus* protein has not been studied yet in a hyperglycaemic microenvironment. The normal microenvironment of cells is a mechanophysiological space provided to healthy and non-diseased cells. In contrast, a hyperglycaemic microenvironment is a small-scale space provided to non-healthy and diabetic cells. Therefore, this study aims to study the efficacy of *O. stamineus* protein extract and protein in accelerating the wound healing activities in both microenvironments.

1.3 Research Goal

1.3.1 Research Objectives

This research work focuses on two main objectives, which are:

1. To study the cell viability of the *O. stamineus* extract and protein on fibroblast cellsunder normal and hyperglycaemic conditions;
2. To study the wound healing activity of the *O. stamineus* extract and protein onfibroblast cells under normal and hyperglycaemic conditions.

1.4 Scope of study

This study is aimed to identify the effectiveness of *O. stamineus* extract and protein in accelerating the wound healing activity of human skin fibroblast cells in the normal and hyperglycaemic microenvironment. The first stage was the preparation of the normal and hyperglycemic condition for the HSF 1184 cells before treated with the various concentrations (7.81, 15.63, 31.25, 62.5, 125, 250, 500 and 1000 μg/mL) and (1.25, 2.5, 5, 10 and 20 ng/mL) of *O. stamineus* ethanolic and protein extract.

Following the treatment, the second scope of this study was to study the cell viability of the *O. stamineus* ethanolic and protein extracts on HSF 1184 cells. In this study, MTT assay was assessed to examine the viability of the cell and the capacity of the treatments in assisting the cell migrating activity against HSF 1184 cells for 72 hours. The final scope of this study was to study the wound healing activity of the *O. stamineus* ethanolic and protein extracts on HSF 1184 cells.

1.5 Significance of the study

In this experiment, the *O. stamineus* leaves extract and protein was chosen because several previous studies have reported the antidiabetic properties of the *O. stamineus* leaf and its potential in treating diabetes (Singh *et al.*, 2015). Previous research has shown that the reduction in blood glucose levels towards diabetic cells may be attributed primarily to the antidiabetic activity of *O. stamineus* (Lokman *et al.*, 2019). Furthermore, it has been reported that the active compounds found in *O. stamineus* have antidiabetic properties, whether acting independently or synergically (Mohamed *et al.*, 2013). Research shows that *O. stamineus* extract may be a promising diabetic wound healing phytomedicine that should be investigated further in preclinical and clinical trials (Abdullah *et al.*, 2020). The antidiabetic properties of the plant can also contribute to the wound healing effect, according to this study.

As a result, the biomedical properties of the *O. stamineus* plant would be a perfect blend of both antidiabetic and wound healing agents. This research can be a breakthrough in explaining the extract and protein source of the *O. stamineus* in treating diabetic wounds since it is a green material, and the herb is widely available and grows readily in the Malaysian climate. This study may provide scientific evidence to the effectiveness of the *O. stamineus* extract and protein in contributing to accelerate the healing of diabetes-related wounds.

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