UNIVERSITI PUTRA MALAYSIA

IN VITRO ANTIOXIDANT, ANTI-DIABETIC, ANTI-INFLAMMATORY, AND WOUND-HEALING PROPERTIES OF Mitragyna speciosa (Korth.) Havil. METHANOLIC EXTRACT

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By

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

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February 2022

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Diabetes mellitus (DM) occurs as the consequence of the destruction of insulin-producing pancreatic beta (β)-cells (type 1 DM) or cell resistance to insulin (type 2 DM). Infections with a slow rate of wound healing are commonly observed in patients with DM. Diabetic foot ulcer is a common complication of DM which imposes high costs for its treatment and management. Chronic wounds in diabetes are associated with impaired angiogenesis, leukocyte function, and fibroblast proliferation. Plant-based remedies such as *Mitragyna speciosa* (*M. speciosa*) have been used by local people in Malaysia as a complementary treatment for various illnesses including lowering blood glucose in diabetic patients. This study aims to determine the *in vitro* antioxidant, anti-diabetic, anti-inflammatory, and wound-healing properties of *M. speciosa* methanolic extract (MSME). The screening of phytochemical compounds in MSME was performed by ultra-high-performance liquid chromatography coupled with traveling-wave ion mobility spectrometry-quadrupole time of flight mass spectrometry (UHPLC-TWIMS-QTOF-MS/MS) analysis. The antioxidant content and scavenging activity of MSME were evaluated by total phenolic content (TPC) assay and total flavonoid content (TFC) assay along with 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) assay and 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, respectively. In addition, the cytotoxicity effects of MSME on RAW264.7 macrophages cells and 3T3-L1 fibroblast cells together with the antioxidative effect of MSME

against oxidative stress in hydrogen peroxide (H_2O_2)-induced 3T3-L1 cells were determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay. The anti-diabetic properties of MSME were studied by measurement of α-amylase enzyme inhibition. Besides, the glucose uptake activity of MSME was determined by the median fluorescence intensity (MFI) of 2-NBDG in 3T3-L1 cells. Anti-inflammatory properties of MSME were determined in LPS-stimulated RAW264.7 cells through secretion of inflammatory mediators including nitric oxide (NO) and cytokines by Griess assay and cytometric bead array (CBA), respectively. The ability of MSME in accelerating cell migration and wound closure was investigated on 3T3-L1 cells by scratch assay. The phytochemical compounds identified in MSME (100 mg/mL) include rutin, epicatechin, quercetin, procyanidin B2, and chlorogenic acid. MSME (1 mg/mL) has lower Total Phenolic Content (TPC) than positive control Pterostilbene (Ptb) (MSME: 167.43±13.50 mg GAE/g sample and Ptb: 230.52±10.92 mg GAE/g sample) but high in TFC (MSME: 347.72±15.97 mg QE/g sample and Ptb: 212.73±17.92 mg QE/g sample). MSME showed relatively similar antioxidant scavenging activity (IC_{50}=4.34 µg/mL) with Ptb (IC_{50}=4.39 µg/mL) in the DPPH assay. Conversely, in the ABTS assay, MSME showed lower antioxidant scavenging activity (IC_{50}=4.25 µg/mL) than Trolox and Ptb (IC_{50}=1.50 and 1.56 µg/mL, respectively). MSME (25, 50, and 100 µg/mL) did not show any toxicity effect on cell survival and protected 3T3-L1 cells from oxidative damage by H_2O_2. Increased inhibition of α-amylase activity (46.39±4.43%) and glucose uptake (MFI: 274.00±8.00) were detected in the 100 µg/mL of MSME suggesting anti-diabetic activity of MSME. MSME was also found to have anti-inflammatory activity through the suppression of NO and cytokine levels in LPS-stimulated RAW264.7 cells. In addition, MSME also induces wound closure in 3T3-L1 cells by accelerating cell migration. MSME may increase glucose uptake, downregulate inflammatory responses of macrophages and subsequently accelerate the process of wound repair which shows promising antioxidant, anti-diabetic, anti-inflammatory, and wound-healing properties. Thus, further study should be conducted to recommend *M. speciosa* as a possible treatment for DM and wound healing.

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

SIFAT *IN VITRO* ANTIOKSIDAN, ANTI-DIABETES, ANTI-RADANG, DAN PENYEMBUHAN LUKA DALAM ESTRAK METANOLIK *Mitragyna speciosa* (Korth.) Havil.

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Diabetes mellitus (DM) berlaku akibat daripada pemusnahan sel beta(β)-pankreas yang menghasilkan insulin (DM jenis 1) atau kerentanan sel terhadap insulin (DM jenis 2). Jangkitan dengan kadar penyembuhan luka yang perlahan biasanya diperhatikan pada pesakit DM. Ulser kaki diabetes adalah komplikasi biasa DM dengan kos rawatan dan pengurusan yang tinggi. Luka kronik pada diabetes dikaitkan dengan gangguan angiogenesis, fungsi leukosit, dan percambahan fibroblas. Ubatan berasaskan tumbuhan seperti *Mitragyna speciosa* (*M. speciosa*) telah digunakan oleh penduduk tempatan di Malaysia sebagai rawatan sampingan penyakit termasuk menurunkan glukosa darah pada pesakit diabetes. Kajian ini bertujuan untuk menentukan sifat *in vitro* antioksidan, anti-diabetes, anti-radang, dan penyembuhan luka ekstrak metanolik *M. speciosa* (MSME). Pemeriksaan kompaun fitokimia dalam MSME dilakukan dengan kromatografi cecair berprestasi tinggi ditambah dengan spektrometri mobiliti ion gelombang perjalanan-kuadrupol masa spektrometri jisim penerbangan (UHPLC-TWIMS-QTOF-MS/MS). Kandungan antioksidan dan aktiviti penangkapan radikal MSME dinilai berdasarkan pengujian kandungan fenolik (TPC) dan ujian kandungan flavonoid (TFC) bersama dengan 2,2'-azino-bis (3-etilbenzothiazolin-6-sulfonik asid) (ABTS) dan 2,2-difenil-1-pikrilhidrazil (DPPH). Di samping itu, kesan sitotoksisiti MSME pada sel makrofaj RAW264.7 dan sel fibroblas 3T3-L1 bersama dengan kesan antioksidatif MSME terhadap tekanan oksidatif pada sel 3T3-L1 yang disebabkan oleh hidrogen peroksida (H_2O_2) ditentukan oleh ujian 3-(4,5-dimetilthiazol-2-yl)-5-(3-carboksimetoksifenil)-2-(4-sulfofenil)-2H-tetrazolium (MTS). Sifat anti-diabetes MSME dikaji dengan pengukuran penampanan

enzim α-amilase. Selain itu, aktiviti pengambilan glukosa MSME ditentukan oleh intensiti pendarfluor median (MFI) 2-NBDG pada sel 3T3-L1. Sifat anti-radang MSME ditentukan dalam sel RAW264.7 yang dirangsang LPS melalui rembesan pengantara keradangan termasuk nitrik oksida (NO) dan sitokin oleh asai Griess dan susunan manik sitometrik (CBA), masing-masing. Keupayaan MSME dalam mempercepat migrasi sel dan penutupan luka dikaji pada sel 3T3-L1 dengan asai calaran. Sebatian fitokimia yang dikenal pasti dalam MSME (100 mg/mL) termasuk rutin, epikatekin, quercetin, procyanidin B2, dan asid klorogenik. MSME (1 mg/mL) mempunyai Kandungan Fenolik Total (TPC) yang lebih rendah daripada kawalan positif Pterostilbene (Ptb) (MSME: 167.43±13.50 mg GAE/g sampel dan Ptb: 230.52±10.92 mg GAE/g sampel) tetapi kandungan TFC yang lebih tinggi (MSME: 347.72±15.97 mg QE/g sampel and Ptb: 212.73±17.92 mg QE/g sampel). MSME menunjukkan aktiviti penangkapan radikal yang serupa (IC_{50}=4.34 µg/mL) dengan Ptb (IC_{50}=4.39 µg/mL) dalam asai DPPH. Sebaliknya, dalam asai ABTS, MSME menunjukkan aktiviti penangkapan radikal yang lebih rendah (IC_{50}=4.25 µg/mL) daripada Trolox dan Ptb (masing-masing IC_{50}=1.50 dan 1.56 µg/mL). MSME (25, 50, dan 100 µg/mL) tidak menunjukkan kesan ketoksikan terhadap kelangsungan hidup sel dan melindungi sel 3T3-L1 dari kerosakan oksidatif yang disebabkan oleh H_2O_2. Peningkatan dalam penghambatan aktiviti α-amilase (46.39±4.43%) dan pengambilan glukosa (MFI: 274.00±8.00) dikesan dalam kepekatan 100 µg/mL MSME yang mencadangkan aktiviti anti-diabetes oleh MSME. MSME juga didapati mempunyai aktiviti anti-radang melalui perencatan tahap NO dan sitokin dalam RAW264.7 LPS-terangsang. Selain itu, MSME juga mendorong penutupan luka pada sel 3T3-L1 dengan mempercepat migrasi sel. MSME dapat meningkatkan pengambilan glukosa oleh sel, menurunkan tindak balas keradangan makrofaj dan seterusnya mempercepat proses pembaikan luka yang menunjukkan sifat antioksidan, anti-diabetes, anti-radang, dan penyembuhan luka yang berpotensi. Oleh itu, kajian lebih lanjut harus dilakukan untuk mengetengahkan *M. speciosa* sebagai kemungkinan bagi rawatan alternatif DM dan penyembuhan luka.

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

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Declaration by the Members of Supervisory Committee

This is to confirm that:

- the research conducted and the writing of the thesis was under our supervision;
- supervision responsibilities as stated in the Universiti Putra Malaysia (Graduate Studies) Rules 2003 (Revision 2012-2013) are adhered to.

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LIST OF ABBREVIATIONS

α	Alpha
β	Beta
ơ	Delta
μ	Mu
°C	Degree celsius
%	Percentage
μg	Microgram
μL	Microliter
μM	Micromolar
$^1O_2$	Oxygen singlet
2-NBDG	2-(N-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl)Amino)-2-Deoxyglucose
ABTS	2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid 3-ethylbenzothiazoline-6-sulphonic acid
$AlCl_3$	Aluminium chloride
AMP	Adenosine monophosphate
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
$C_2H_2O_2K$	Potassium acetate
CBA	Cytometric bead array
CCL	Chemokine ligand
CE	Collision energies
CID	Collision-induced-dissociation

COX	Cyclooxygenase
Da	Dalton
DAMPS	Danger-associated molecular patterns
DM	Diabetes mellitus
DMEM	Dulbecco's modified Eagle's medium
DNA	Deoxyribonucleic acid
DNSA	Dinitrosalicylic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
EGF	Epidermal growth factor
EPC	Endothelial progenitor cell
ESI	Electrospray ionisation
FBS	Fetal bovine serum
g	Gram
GAE	Gallic acid equivalent
GLP	Glucagon-like peptide
GLUT4	Glucose transporter 4
h	Hour
H_2O_2	Hydrogen peroxide
HDMSE	High-definition MSE
HE	High-energy
IFN	Interferon
IL	Interleukin
iNOS	Inducible nitric oxide synthase
JAK	Janus kinase
$K_2S_2O_8$	Potassium persulfate

kV	Kilovolt
L	Liter
LE	Low-energy
LOX	Lipoxygenase
LPS	Lipopolysaccharide
M	Molar
m/z	Mass-to-charge ratio
M1	Classically activated
M2	Alternatively activated
MAPKs	Mitogen-activated protein kinase
MCP	Monocyte chemoattractant protein
mg	Milligram
min	Minute
mM	Millimolar
MFI	Median fluorescence intensity
mL	Milliliter
MS/MS	Mass spectrometry
MSME	*M. speciosa* methanolic extract
MTS	3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
MW	Molecular weight
NA_2CO_3	Sodium carbonate
NAD	Nicotinamide adenine dinucleotide

NF-κB	Nuclear factor-kappa-light-chain-enhancer of activated B cells
NLRP3	NOD-like receptor protein 3
nm	Nanometre
NO	Nitric oxide
NOD	Nucleotide-binding domain
$O_2^{-\cdot}$	Superoxide
$OH^{\bullet}$	Hydroxyl
$ONOO^-$	Peroxynitrite
PAMPs	Pathogen-associated molecular patterns
PBS	Phosphate-buffered saline
PDGF	Platelet-derived growth factor
pg	Picogram
PGE-2	Prostaglandin E2
PKC	Protein kinase C
ppm	Parts per million
PRRs	Pattern-recognition receptors
Ptb	Pterostilbene
QE	Quercetin equivalent
QTOF	Quadrupole time of flight
ROS	Reactive oxygen species
s	Second
SEM	Standard error mean
STAT	Signal transducer and activator of transcription
T1DM	Type 1 DM

T2DM	Type 2 DM
TFC	Total flavonoid content
Th	T-helper
TLR	Toll-like receptor
TNF	Tumour necrosis factor
TPC	Total phenolic content
TWIMS	Traveling-wave ion mobility spectrometry
UHPLC	Ultra-high-performance liquid chromatography
w/v	Weight per volume

CHAPTER 1

INTRODUCTION

1.1 Background

Diabetes mellitus (DM) is a metabolic disorder characterized by chronic hyperglycemia with defects in carbohydrate, fat, and protein metabolism. DM arises due to the destruction of insulin-producing pancreatic beta (β)-cells (type 1 DM) or cell resistance to insulin (type 2 DM). Another type of DM is known as gestational diabetes which can occur among women during pregnancy (Lal, 2016). The symptoms of DM can be varied including the most classical symptoms such as frequent urination, extreme feeling of thirst, increased appetite, loss of weight, blurry vision, and fatigue (Ramachandran, 2014). All types of DM symptoms can be controlled to reduce the high risk of severe complications such as cataracts, retinopathy, neuropathy, skin infection, ischemic heart disease, hypertension, ketoacidosis, nephropathy, and stroke (Lal, 2016). Although at present there is no known cure for diabetes, some treatments can help reduce the symptoms of DM and prevent the development of severe complications. For example, type 1 DM (T1DM) patients are treated with regular insulin shots, association with a special diet, yoga, and exercise, while patients with type 2 DM (T2DM) are commonly treated with tablets, exercise, and a special diet, but occasionally insulin injections are also necessary (Lal, 2016).

Infections with a slow rate of wound healing are commonly observed in patients with DM. A diabetic foot ulcer is a common complication of DM which imposes a high cost for its treatment and management (Alexiadou & Doupis, 2012). Chronic wounds in diabetes are associated with impaired angiogenesis, leukocyte function, and fibroblast proliferation (Okonkwo & Dipietro, 2017). Wound and skin injuries are conditions that lead to high susceptibility to infections and inflammation. The acute inflammatory process is mediated and controlled by phagocytic innate immune cells particularly neutrophils and macrophages (Serra et al., 2017). Activation of these cells is crucial for the elimination of bacterial infection. During the inflammatory process, these cells produce various pro-inflammatory mediators and several cytokines (Zhang & An, 2007). Due to the ability of these cells in causing or worsening tissue damage, downregulation of their inflammatory factors is required for the resolution of inflammation and subsequently leads to tissue recovery (Serra et al., 2017).

In the past, many studies investigated various plants which showed to be a basis for the discovery of novel drug compounds and act as plant-based

medicines which have greatly benefaction to the healthcare industry (Etukudoh, Uchejeso, & Etim, 2021). Traditional medicine including herbal medicine has become part of the practice by people throughout the world to improve human health and well-being (Kim Sooi & Lean Keng, 2013). In Malaysia, the practices of herbal medicines in treating various illnesses are increasingly popular among the community and also becoming significant to the public. This is due to the belief that herbal products do not contain any destructive chemicals and exhibit lesser side effects when compared to commercially available conventional medicine (Kim Sooi & Lean Keng, 2013).

A previous study has documented that diabetic patients chose to consume indigenous herbs and traditional medicine to treat diabetes which include amaranth leaves (*Amaranth species*), hare lettuce (*Sonchus luxurians*), nightshade leaves (*Solanum villosum millers*), spider plant leaves (*Gynandropsis gynandra*), and okra pods (*Abelmoschus esculentus*) (Kasole, Martin, & Kimiywe, 2019). Besides, a traditional plant originating from Asia known as bitter melon (*Momordica charantia*) has been consumed to treat diabetes and its related complications. It is reported that the structure (polypeptide-p) and the mechanism of action of bitter melon are similar to insulin (Cefalu, Stephens, & Ribnicky, 2011).

A plant-based remedy including *Mitragyna speciosa* (*M. speciosa*) or Kratom leaf which can mostly be found in Southeast Asia has been used by local people in Malaysia as a complementary treatment for various illnesses including lowering blood glucose in diabetic patients (Idayu et al., 2011). In Thailand, *M. speciosa* has also been traditionally used by natives to treat diabetes, though there is a lack of scientific evidence on its efficacy (Purintrapiban et al., 2011). Therefore, this study was designed to analyze antioxidant levels of MSME by evaluating its TPC and TFC along with DPPH and ABTS radical scavenging activity as well as its cytoprotective effect on H_2O_2-induced 3T3-L1 cells; anti-diabetic by determining the inhibition of alpha-amylase enzyme and glucose uptake activity by 3T3-L1 cells; anti-inflammatory through an assessment on the production of pro-inflammatory mediators including NO and cytokines in LPS-stimulated RAW264.7 cells, and subsequently its effect on wound healing activity by wound scratch assay in 3T3-L1 cells. The methanolic extraction method was performed in the present study to obtain the crude extract of *M. speciosa.* Methanol was used as a solvent in this study as a previous study revealed the highest extraction yield in methanolic extract when compared to distilled water, ethanol, chloroform, dichloromethane, and acetone extracts, suggesting better extraction efficiency with highly polar solvents such as methanol (Truong et al., 2019).

1.2 Objectives

1.2.1 General objective

- To determine the *in vitro* antioxidant, anti-diabetic, anti-inflammatory, and wound healing properties of *M. speciosa* methanolic extract (MSME).

1.2.2 Specific objectives

- To evaluate the total phenolic and flavonoid content of MSME by TPC and TFC assays, and its antioxidant activity through assessment of radical scavenging activity by DPPH and ABTS assays.
- To investigate the cytotoxicity effect of MSME and its protective effect against free radical hydrogen peroxide (H_2O_2) through evaluation of cell viability.
- To investigate the anti-diabetic activity of MSME through assessment of alpha (α)-amylase enzyme inhibition and glucose uptake capability in the 3T3-L1 fibroblast cell line.
- To determine the anti-inflammatory properties of MSME in LPS-stimulated macrophages RAW264.7 cell line through evaluation of pro-inflammatory mediators such as nitric oxide (NO) and cytokines.
- To study the effects of MSME on wound closure through the observation of 3T3-L1 cell migration by scratch wound healing assay.

1.3 Hypothesis

M. speciosa methanolic extract (MSME) possesses antioxidative effects, increases cellular glucose uptake, α-amylase enzyme degradation, downregulates inflammatory responses of macrophages, and accelerates the process of wound repair.

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