ANTIOXIDANT AND ANTIDIABETIC ACTIVITIES OF PROTEINS FROM *Moringa oleifera*

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DEDICATION

This thesis is dedicated to my father, who taught me that the best kind of knowledge to have is that which is learned for its own sake. It is also dedicated to my mother, who taught me that even the largest task can be accomplished if it is done one step at a time.

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ABSTRACT

Moringa oleifera is among the highly reported medicinal plant with abundant health benefits mostly shown by its leaf and seed. Currently, research on antioxidant and antidiabetic activities of the plant have been focused on its aqueous and solvent extracts. In fact, study on proteins from this plant is still limited. Furthermore, the discovery of Moringa protein's antioxidant and antidiabetic activity supports its potential application in the medical sector. Hence, protein from Moringa's leaf, seed and petiole were extracted before their antioxidant and antidiabetic activity were established. Determination of the best protein extraction method for different parts of Moringa revealed that all three parts were best extracted using Tris buffer with incorporation of dithiothreitol (DTT) and phenylmethylsulfonyl fluoride (PMSF). Next, 1D proteomics using Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) and in-solution digestion prior to Liquid Chromatography–Mass Spectrometry (LC-MS/MS) analysis were subsequently done to establish the protein profiling of different plant parts. There were 144, 125, and 122 proteins identified from the leaf, seed and petiole, respectively. Interestingly, few peptides responsible for antioxidant and anticancer activity were identified. These proteins include maternally expressed gene 5, glutathione biosynthesis and catalase from the leaf, myrosinase, thioglucoside glucosidase and peroxidase in the seed while catalase, peroxidase, and glutathione transferase from the petiole. Furthermore, the antioxidant ability of both protein and crude extracts from Moringa's leaf, seed and petiole was determined via Ferric Reducing Antioxidant Power (FRAP) and 2, 2-diphenyl-1-picryl-hydrazyl-hydrate (DPPH) free radical assay. Since the protein extract of leaf and petiole exhibited higher reducing activity compared to the crude extract, potential pharmacological activity of the protein extract was anticipated. Thus, the antidiabetic activity of the protein extracted from leaf, petiole, and seed were further studied using α-amylase and α-glucosidase inhibition assays. The highest α-amylase inhibition activity of 96% was conferred by the seed while highest α-glucosidase inhibition activity of 92% was conferred by the leaf. Moreover, a high potential antidiabetic agent namely Transketolase (TKT) enzyme was identified in the petiole. In silico analysis of TKT indicated it to be a thermostable protein upon molecular dynamics (MD) simulation at 310 K, 373 K, 423 K, 453 K, and 473 K. The findings of this study provide proteomic analysis of *M. oleifera's* leaf, seed and petiole. These protein extracts were proven to possess both antioxidant and antidiabetic activity and further supported by the protein profiling results. In fact, bioinformatics findings on TKT provide meaningful insight towards potential drug development as it pose potential roles in combating diabetes.

ABSTRAK

Moringa oleifera merupakan antara tumbuhan perubatan yang dilaporkan memiliki pelbagai khasiat terutamanya dalam daun dan benihnya. Buat masa ini, kajian tentang aktiviti antioksidan dan antidiabetik melibatkan Moringa tertumpu kepada ekstrak air dan pelarut sahaja. Bahkan, kajian tentang protein dari tumbuhan ini masih terhad. Tambahan pula, penemuan aktiviti antioksidan dan antidiabetik protein Moringa menyokong penggunaannya dalam sektor perubatan. Oleh itu, protein daripada daun, benih, dan petiol Moringa telah diekstrak keluar sebelum aktiviti antioksidan dan antidiabetik mereka dilaksanakan. Penentuan kaedah pengekstrakan protein terbaik untuk bahagian tumbuhan Moringa mendedahkan bahawa ketiga-tiga bahagian itu paling baik diekstrak menggunakan penampan Tris dengan penambahan dithiothreitol (DTT) dan phenylmethylsulfonyl fluorida (PMSF). Seterusnya, proteomik 1D menggunakan Natrium Dodesil Sulfat Poliakrilamida Gel Elektroforesis (SDS-PAGE) dan analisis Kromatografi cecair–spektometri jisim (LC-MS/MS) dilakukan untuk mewujudkan profil protein bahagian-bahagian tumbuhan. Sebanyak 144, 125, dan 122 protein telah berjaya dikenalpasti dari daun, benih dan petiol. Menariknya, beberapa protein dan peptida yang bertanggungjawab terhadap antioksidan dan antikanser telah dikenal pasti. Protein-protein berkenaan adalah adalah *protein maternally expressed gene 5*, biosintesis glutation dan katalase dari daun, myrosinase, thioglukosida glukosidase dan peroksidase dalam benih manakala katalase, peroksidase, dan glutation transferase dari petiol. Tambahan pula, keupayaan antioksidan kedua-dua protein dan ekstrak mentah dari daun, benih dan petiol Moringa telah ditentukan melalui ujian antioksida; *ferric reducing antioxidant power* (FRAP) dan 2, 2-diphenyl-1-picryl-hydrazyl-hydrazyl-hydrazyl-hydrate (DPPH). Memandangkan ekstrak protein daun dan petiol mempamerkan aktiviti antioksida yang lebih tinggi berbanding dengan ekstrak mentah, potensi aktiviti farmakologi ekstrak protein sangat diharapkan. Oleh itu, aktiviti antidiabetik protein yang diekstrak dari daun, benih, dan petiol telah dikaji lagi menggunakan ujian perencatan sebaran glukosa, α-amilase dan α-glukosidase. Aktiviti perencatan α-amilase tertinggi sebanyak 96% telah ditunjukkann oleh benih manakala aktiviti perencatan α-glukosidase tertinggi sebanyak 92% telah ditunjukkan oleh daun. Selain itu, ejen antidiabetik berpotensi tinggi iaitu enzim Transketolase (TKT) buat pertama kalinya telah dikenal pasti dalam petiol. Analisis in-siliko menunjukkan TKT adalah protein stabil-suhu berdasarkan simulasi dinamik molekul (MD) pada 310 K, 373 K, 423 K, 453K, dan 473K. Secara keseluruhannya, kajian ini memaparkan penemuan analisis proteomik daun, benih dan petiol *M. oleifera*. Ekstrak protein ini terbukti mengandungi kedua-dua aktiviti antioksidan dan antidiabetik yang kemudiannya disokong oleh hasil pemprofilan protein. Malah, kajian bioinformatik TKT memberi pendedahan ke arah pembangunan ubat kerana TKT dilihat berpotensi untuk melawan penyakit diabetes.

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LIST OF ABBREVIATIONS

1D	-	One dimensional
2-DE	-	Two-dimensional electrophoresis
3D	-	Three-dimensional
APS	-	Ammonium persulfate
BSA	-	Bovine serum albumin
CHAPS	-	3-(3-Cholamidoprpyl)dimethyammonia-1-Propanesulfonic Acid
CBB	-	Coomassie Brilliant Blue
cm	-	Centimetre
DNA	-	Deoxyribonucleic acid
DPP-4	-	Dipeptidyl Peptidase-4
DPPH	-	2, 2-diphenyl-1-picrylhydrazyl.
DTT	-	Dithiothreitol
EDTA	-	Ethylenediaminetetraacetic acid
et al.	-	And friends
$FeSO_4.7H_2O$	-	Ferrous Sulphate Heptahydrate
FRAP	-	Ferric Reducing Antioxidant Power
GO	-	Gene Ontology
GROMACS	-	GROningen MAchine for Chemical Simulations
HCl	-	Hydrochloric acid
IAA	-	Indole-3-acetic acid
IEF	-	Isoelectric focusing
IPG	-	Immobilised pH gradient
I-TASSER	-	Iterative Threading Assembly Refinement
KPO_4	-	Potassium phosphate
LC-MS/MS	-	Liquid Chromatography Tandem Mass Spectrometry
MD	-	Molecular Dynamic
Mg	-	Magnesium
NCBI	-	National Centre for Biotechnology Information
PDB	-	Protein Data Bank

PPP	-	Pentose Phosphate Pathway
QB	-	Quenching buffer
Rg	-	Radius of Gyration
RMSD	-	Root-Mean-Square Deviation
RMSF	-	Root-Mean-Square Fluctuation
RNA	-	Ribonucleic acid
ROS	-	Reactive Oxygen Species
RSA	-	Radical Scavenging Activity
SDS	-	Sodium Dodecyl Sulfate
SDS-PAGE	-	Sodium Dodecyl Sulfate- Polyacrylamide Gel Electrophoresis
SEM	-	Standard Error Mean
T5X	-	Xylulose-5-Phosphate
TCA	-	Trichroloacetic acid
TEMED	-	Tetramethylethylenediamine
TKT	-	Transketolase
TPP	-	Thiamine pyrophosphate
TPTZ	-	2, 4, 6-tri-(2-piridyl)-s-triazine
Tris HCL	-	Tris Hydrochloride
UTM	-	Universiti Teknologi Malaysia

LIST OF SYMBOLS

α	-	Alpha
A_{595}	-	Absorbance at 595 nanometre
β	-	Beta
°C	-	Degree Celsius
<	-	Less than
%	-	Percent
G	-	Gram
K	-	Kelvin
kDa	-	Kilo Dalton
µg	-	Microgram
µg/mL	-	Microgram per millilitre
µg/g	-	Microgram per gram
µL	-	Microliter
M	-	Moles
mg	-	Milligram
mL	-	Millilitre
mM	-	Milli Molar
m/z	-	Mass/Charge
ns	-	Nanosecond
Rpm	-	Rotary per minute
%	-	Percent
U	-	Unit
V	-	Voltage
v/v	-	Volume per volume
w/v	-	Weight per volume

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CHAPTER 1

INTRODUCTION

1.1 Problem Background

Scientists of the world have made splendid discovery on numerous therapeutic effects of crude extracts from vast list of herbal medicinal plants (Khan *et al.*,2012). The antioxidant and antidiabetic properties of those plant extracts are among the most studied therapeutic traits by the researchers. Moreover, the antioxidant traits of the plants are highly associated with good health and disease prevention while possessing antidiabetic properties is a major weapon against diabetes, chronic disease associated with obesity and major cause of death affecting millions of lives (Johansen *et al.*,2005; Kaneto *et al.*,1999). Interestingly, tremendous findings related to antioxidant and antidiabetic activity of medicinal plants are only restricted to their crude extract (Bakirel *et al.,* 2008; Azzahra *et al.,* 2012; Godwill *et al.,* 2016). Critical studies on the activity of protein extracted from the plants are yet to be done. Protein is a highly essential nutrient needed by our body (Colovos and Yeates, 1993). It is not only found in plants and other living organisms but is commonly incorporated in marketed drugs mainly due to its special binding ability. Special protein binding ability is contributed by its short and simple structure (Hartmann and Meisel, 2007). In fact, due to protein's high specificity and reactivity towards its substrate and ligand, the enzyme-substrate binding and consequently reaction were effectively achieved (Konig et al.,1994; Obiol-Pardo and Rubio-Martinez, 2009). Hence, detailed study on their therapeutic activities including antioxidant and antidiabetic ability is a must.

Moringa oleifera (Moringaceae) is a herbal medicinal plant synonyms with various vernacular names such as drumstick tree, horseradish tree, miracle tree and it is locally known as "kelor" (Anwar and Bhanger, 2003). This native plant of India is currently cultivated in tropical and subtropical counties including Malaysia (Saini *et al.*, 2013). Similar to coconut tree, this plant is famous for its multipurpose use where

every part of this plant possesses different function and properties (Amaglo *et al.*, 2010; Bennett *et al.*, 2003). The leaf, seed and stems of this perennial herb are demanded for human and animal consumption according to their scientifically proven pharmacological properties such as antioxidant and antidiabetic activity (Aregheore, 2002; Rahman *et al.*, 2009; Sánchez *et al.*, 2006; Sreelatha *et al.*, 2011). However, those reported studies focused on the water solubility and methanolic extract of the plant in which its protein extract activity is yet to be tested. Prominently, this plant is claimed to have higher protein content compared to eggs and yogurt. Hence, detailed study on its protein activity specifically on its antioxidant and antidiabetic ability is a brilliant option in order to unveil their full potential in benefiting mankind through their application in pharmaceutical sector.

One of the proteins that may play a role in antidiabetic activity is transketolase (TKT). TKT protein plays a significant role to reversibly convert sugar and generate antioxidant NADPH in pentose phosphate pathway (PPP). TKT enzyme activity was found to be lower in diabetic patients and its activation would effectively inhibits biochemical pathways responsible for diabetic implications (Alam, Riaz, and Akhtar, 2011; Berrone *et al.*, 2006; Hammes *et al.*, 2003). Previous study reported by Wang *et al.*, (2016) has identified TKT protein in Moringa grown in China. This TKT has high potential as a target in diabetic disease management. Additionally, bioinformatics as a medium towards comprehensive understanding on the plant's protein information and interaction would be beneficial. Protein-protein, protein-ligand and even protein-substrate interaction can be explored by means of bioinformatics. Hence, in silico analysis including molecular dynamic (MD) simulation on the targeted protein; TKT from Moringa serves as a functional approach to efficiently predict its structure and activity at different temperature.

1.2 Problem Statement

Enormous studies were reported on the pharmacological traits of phytochemical compounds present in *M. oleifera*. However, detailed study on its protein extract are still lacking. Even though Wang *et al.*,(2016) had reported on the

protein profiling of *M. oleifera* from China, the geographical differences is expected to lead to variation in the identified protein. Furthermore, protein from the petiole tissue of this plant is yet to be profiled.

While researchers are currently digging into the ability of proteins and peptides in aiding the health sector, research on proteins and peptides from medicinal plant are still lacking. In fact, the antidiabetic and antioxidant activity of the protein extracted from this plant is yet to be determined. This is contrary to the phenomenal findings of the antioxidant and antidiabetic activity of the plant's crude extract (Idakwoji *et al.*, 2016; Ilyas *et al.*, 2015).

Combination of the high affinity and efficiency of the protein technology and the valuable antidiabetic properties of *M. oleifera* are expected to provide exquisite platform in the new antidiabetic drug development such as TKT. This technology can be foreseen replacing the awful synthetic medicines currently available in the market. Therefore, potential antioxidant and antidiabetic activity of the protein extract from this plant including the TKT structure and activity should be studied extensively.

1.3 Significance of Study

Medicinal plants are abundant in nature and contains rich source of bioactive compounds. Additionally, proteins and peptides from medicinal plants offer low to zero risk of rejection and side effects. They are free from animal cholesterol and suit the vegetarians. Furthermore, unpleasant scenario the pharmacological industries facing due to negative side effects from synthetic drugs is calling for new remedies. Thereupon, extensive study on antioxidant potential and diabetes based on proteomic approach using protein extracts and bioactive peptides from food sources such as plant with multiple proven pharmacological benefits is a brilliant preference.

Despite the large number of studies on the application of *M. oleifera* extract, reports on pharmacological properties of the proteins and peptides from this plant is still absent. In addition, determination of the properties and identities of proteins from

Malaysian *M. oleifera* possess major contribution to the body of knowledge especially when related to the activity and content of this miracle medicinal plant. A better understanding on its antioxidant and antidiabetic activity can be accomplished. Hence, it could be further applied to reap its benefit to mankind. Furthermore, the TKT enzyme extracted from this plant is a high potential drug specifically for antidiabetic treatment to replace the existing synthetic alternatives.

1.4 Research Objectives

The four main objectives of this study were:

(a) To profile proteins and peptides with medicinal value from the leaf, petiole, and seed of *M. oleifera.*

(b) To analyze the antioxidant activity between the protein and crude plant extract of *M. oleifera* (leaf, petiole, and seed).

(c) To analyze the antidiabetic properties of protein extract from *M. oleifera* (leaf, petiole, and seed) via α-amylase and α-glucosidase inhibitory activity.

(d) To predict thermostability and docking mechanism of selected proteins with antidiabetic potential from profiled petiole protein.

1.5 Scope of Study

Protein from three parts of *M. oleifera* namely leaf, petiole, and seed were extracted using Tris-HCl buffer before their protein quality and quantity was determined via Bradford assay and One Dimensional Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis (1D SDS-PAGE) respectively. Protein profiling of all the three parts of *M. oleifera* was studied using one dimensional (1D) polyacrylamide gel electrophoresis (PAGE), In-solution digestion and LC-MS/MS analysis for the identification of peptides with potential antioxidant, antidiabetic and anticancer activity. Then, the antioxidant activity according to FRAP and DPPH assays of both protein and crude extract from the plant was also determined. Next, the

antidiabetic potential of the protein extract from leaf, petiole, and seed using α-glucosidase and α-amylase inhibition assay was successfully done. Finally, thermostability and docking mechanism prediction of selected TKT protein with antidiabetic potential from profiled petiole protein was successfully simulated. This was done using bioinformatics tools of Autodock for molecular docking and GROningen MAChine for Chemical Simulations (GROMACS) software version 5.0.4 for molecular simulation at 310 K, 373 K, 423 K, 453K, and 473 K.

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LIST OF PUBLICATIONS

Indexed Journal

1. Zulkifli Z. A. and Rahmat Z., Evaluation of effective protein extraction procedure to profile petiole of *Moringa oleifera*, Plant Science Today. 7(2):214–218 (2020). **(Indexed by Web of Science and SCOPUS)**

2. Zulkifli Z. A. and Rahmat Z., Protein Antioxidant Capacity from *Moringa Oleifer*a Fresh and Commercialised Leaf, *Biosci, Biotech. Res. Asia*. 17(1), 155-161 (2020). **(Indexed by Clarivate)**

Non-Indexed Conference

1. Oral Presenter – AFOB Malaysia Chapter International Symposium (2019)
2. Oral Presenter – International Conference on Biosciences and Medical Engineering (2019)
3. Oral Presenter – International Postgraduate Symposium in Biotechnology (2019)
4. Oral Presenter – International Graduate Conference on Engineering, Science, and Humanities (2018)

Book Chapter

1. Zulkifli Z. A., Ng M. L. and Rahmat Z. (2020). One-Dimensional Protein Electrophoresis for the Characterisation of Malaysian Medicinal Plants. In Abdul-Wahab and Dzulkarnain (Eds), Current Techniques in Protein Sciences (pp 27-36). Universiti Teknologi Malaysia: Penerbit UTM Press.

2. Zulkifli Z. A. (2018). Herbal Plant in Livestock Nutrition. In Rahmat and Nik-Malek (Eds), Plant Science and Its Application (pp 191-208). Universiti Teknologi Malaysia: Penerbit UTM Press.