BIOACTIVE POLYPHENOLS FROM *ANACARDIUM OCCIDENTALE* LINN LEAVES AND THEIR EFFECTS ON A-AMYLASE AND DIPEPTIDYL PEPTIDASE IV ACTIVITIES

BY

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ABSTRACT

Anacardium occidentale Linn. (*A. occidentale* L.) or cashew tree is one of the famous tropical plants among various ethnics in Malaysia. Its leaves are commonly consumed as *ulam* in Southeast Asia. Its leaves possess an excellent source of bioactive compounds, which are associated with numerous health benefits, including antidiabetic potency for type 2 diabetes mellitus (T2DM). The management strategies for T2DM include maintaining the postprandial blood glucose level through the inhibition of α-amylase and to prevent the hydrolysis of glucagon-like peptide-1 (GLP-1) through the inhibition of dipeptidyl peptidase IV (DPPIV). The DPPIV is a serine protease that localizes on cell surfaces and responsible for the rapid degradation of incretins such as a gastric inhibitory peptide, whilst α- amylase is served as the necessary digestive enzymes by involving in the breaking of long-chain carbohydrates into small pieces before absorption in the intestine. In this study, free, soluble ester and insoluble bound phenolic fractions from young and mature leaves of *A. occidentale* L. were extracted and analyzed for their phenolic compounds concentration using high-performance liquid chromatography (HPLC). Subsequently, all fractions were investigated for their inhibitory effects on α-amylase and DPPIV enzyme activities. Both free (72.45 ± 3.6%) and soluble ester (83.40 ± 4.7%) phenolic fractions in the mature leaves extracts had significantly demonstrated higher α-amylase inhibitors than the young leaves. Likewise, soluble ester (4.09 ± 0.34 μg/ml) and insoluble bound (4.87 ± 0.32 μg/ml) phenolic fractions in the mature leaves extracts were significantly more effective in inhibiting DPPIV than the young leaves. As for fractions comparison, insoluble bound derived from the young leaves extract was a more potent α-amylase inhibitor than free and soluble ester phenolic fractions ($p<0.001$). Soluble ester and insoluble bound phenolic fractions showed stronger inhibitors of DPPIV than the free phenolic ($p<0.001$), based on the maturity of the leaves. The interaction between fraction and maturity were significantly different in both young and mature leaves of *A. occidentale* L. according to the α- amylase, and DPPIV inhibition analyses ($p<0.001$). In conclusion, this study showed that *A. occidentale* L. leaves extracts possessed inhibition properties in α- amylase and DPPIV activities, which further may potentially be exploited in clinical study as an antidiabetic for type 2 diabetes mellitus.

خلاصة البحث

إن نبات الأناكارديوم أوكسيدينتالي لين (*Anacardium occidentale* Linn.)، المعروف بشجرة الكاجو، نبات استوائي معروف بين مختلف الأعراق في ماليزيا. تستهلك أوراقها في جنوب شرق أسيا بصورة عامة كواقي. تعتبر أوراق هذه الشجرة مصدرا ممتازا للمركبات النشطة بيولوجيا، والتي ترتبط بالعديد من الفوائد الصحية مثل خواصها المضادة لمرض السكري من النمط الثاني (T2DM). تشمل استراتيجيات إدارة داء السكري الحفاظ على مستوى السكر في الدم بعد الأكل من خلال تثبيط إنزيم ألفا–الأميليز ومنع التحلل المائي للجلوكاجون مثل الببتيد–1 (GLP-1) بواسطة تثبيط دي ببتيل ببتيداز–4 (DPPIV)، وهو عبارة عن بروتياز سيري يتواجد على أسطح الخلايا و مسؤول عن التحلل السريع لأنسيترين مثل الببتيد المثبط للمعدة. بينما يتم عمل إنزيما ألفا–الأميليز كأنزيمات هضمية ضرورية من خلال عملية تحطيم الكربورهيدرات إلى أجزاء صغيرة قبل هضمها وامتصاصها في الأمعاء.تم في هذه الدراسة استخلاص وتحليل إسترات حرة قابلة للذوبان وفينولات مرتبطة غير قابلة للذوبان من أوراق شجرة الكاجو الصغيرة والكبيرة. للتحقق من تراكيز مركبات الفينول باستخدام الكروماتوغرافيا السائلة عالية الأداء (HPLC). في وقت لاحق تم التحقق من التأثير المثبط لجميع الأجزاء على أنشطة الأنزيمات ألفا أميليز و DPPIV. أظهر كل من الأجزاء الفينولية الحرة (72.45±3.6٪) والإسترات القابلة للذوبان (83.40±4.7٪) في مستخلصات الأوراق الكبيرة تثبيطا أكبر لإنزيم ألفا أميليز وبشكل ملحوظ مقارنة بالأوراق الصغيرة. وبالمثل كانت الإسترات الحرة القابلة للذوبان (4.09±0.34 ميكروغرام/مل) والإسترات المرتبطة غير القابلة للذوبان (4.87±0.32 ميكروغرام/مل) للأجزاء الفينولية من مستخلصات الأوراق الكبيرة أكثر فاعلية في تثبيط DPPIV مقارنة بالأوراق الصغيرة. أما بالنسبة للمقارنة بين الأجزاء، فإن الإسترات المرتبطة غير القابلة للذوبان المشتقة من مستخلصات الأوراق الصغيرة كانت أكثر تثبيطا لألفا أميليز بشكل أكثر فعالية مقارنة بالإسترات الحرة القابلة للذوبان من الأجزاء الفينولية ($p<0.001$). إضافة إلى ذلك أظهرت الإسترات القابلة للذوبان والأجزاء الفينولية المرتبطة غير القابلة للذوبان تثبيطا أقوى لإنزيم DPPIV مقارنة بالفينولات الحرة ($p<0.001$)، على حسب نضج الأوراق. نتج أيضا عن التفاعل بين الأجزاء ومستوى نمو الأوراق اختلافا كبيرا في كل من الأوراق الصغيرة والكبيرة لشجرة الكاجو على تحليل تثبيط إنزيمات ألفا أميليز وDPPIV، ($p<0.001$). ختاما أظهرت هذه الدراسة أن مستخلصات شجرة الكاجو تمتلك خصائص تثبيط في أنشطة ألفا–الأميليز و DPPIV، والتي قد يتم استغلالها في العمليات السريرية لإدارة داء السكري من النمط الثاني.

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APPROVAL PAGE

I certify that I have supervised and read this study and that in my opinion, it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a thesis for the degree of Master of Science (Biotechnology)

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DECLARATION

I hereby declare that this thesis is the result of my own investigations, except where otherwise stated. I also declare that it has not been previously or concurrently submitted as a whole for any other degrees at IIUM or other institutions.

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This thesis is dedicated for my source of inspiration which is my beloved husband, parents and daughter; may Allah SWT grant them jannatul-firdaus.

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LIST OF SYMBOLS

>	Substitution
g	Gravitational force
ml	Milliliter
µl	Microlitre
ºC	Degree Celsius
%	Percentage/ proportion
≥	Greater than or equal to
ng/µl	Nanogram per litre
U/ml	Units per milliliter
mg	Milligram
±	Plus-minus
µM	Micromolar
*	Asterisk
µg	Microgram
α	Alpha
β	Beta
hr	Hour
kDa	Kilodalton
Kg/m^2	Kilogram per square meter
L	Liter
min	Minute

LIST OF ABBREVIATIONS

ANOVA	Analysis of variance
BMI	Body mass index
DMSO	Dimethylsuphoxide
DNA	Deoxyribonucleic acid
DPPH	2,2-diphenyl-1-picrylhydrazyl
DPPIV	Dipeptidyl peptidase IV
e.g.	exempli gratia; for example
et al.	et alia; and others
FTIR	Fourier transform infrared spectroscopy
GAE	Gallic acid equilibrium
GC-MS	Gas chromatography- mass spectrometry
GDM	Gestational diabetes mellitus
GIP	Gastric inhibitory peptide
GLP-1	Glucagon like peptide- 1
H_2O	Water
HbA1c	Hemoglobin A1c
HCl	Hydrochloric acid
HPLC	High performance chromatography
LC-MS	Liquid chromatography- mass spectrometry
M	Molarity
MeOH	Methanol
MOH	Ministry of health
mRNA	messenger Ribonucleic acid
N	Normality
NaOH	Sodium hydroxide
NHMS	National Health Morbidity Survey
nm	Nanometers
NMR	Nuclear Magnetic resonance
PFTE	Polytetrafluoroethylene
R^2	Coefficient of determination
Rpm	Revolution per minute
SEM	Standard error of means
T2DM	Type 2 Diabetes Mellitus
UV-Vis	Ultraviolet-visible
v/v	Volume per volume
WHO	World health organization

CHAPTER ONE

INTRODUCTION

1.1 BACKGROUND

Diabetes mellitus is one of the major health problems that affect millions of people worldwide. It is projected that 642 million people aged 20- 79 years around the globe will suffer from diabetes by 2040 (Ogurtsova et al., 2017). In Malaysia, since the first National Health Morbidity Survey (NHMS I) until recent NHMS V, the prevalence of diabetes mellitus among adult population has markedly increased from 6.3% in 1986, 8.2% in 1996, 11.6% in 2006 and 15.2% in 2011 to 17.5% in 2015 (IPH, 1997; Kementerian Kesihatan Malaysia 2015). Besides, diabetes also becomes one of the leading causes of death globally (Ogurtsova et al., 2017). In 2012, it was reported that diabetes has caused 1.5 million (2.7%) deaths (World Health Organization, 2016).

The management of diabetes involves continuous medical care with the number of factors risk reduction strategies including lifestyle modifications and treatments with synthetic hypoglycaemic drugs. Yet, it is estimated that 70-80% of the world populations still depending heavily on alternative medicine such as traditional herbs to encounter their primary health care needs, including treatments for diabetes, particularly in rural areas of developing states (World Health Organization, 2002). For example, plants such as *Aloe vera*, *Andrographis paniculate* (Green chiretta), *Centella asiatica* (Asiatic pennywort), *Curcuma longa* (Turmeric) and *Anacardium occidentale* (Cashew) have been demonstrated to possess antidiabetic properties (Esimone et al., 2001; Mustaffa et al., 2011; Obaineh, 2013), owing to the existence of bioactive

compounds that act as antidiabetic agents such as carotenoids and polyphenols (Shukri et al., 2011).

1.2 PROBLEM STATEMENT AND ITS SIGNIFICANCE

Medicinal plants are good sources as alternative or complementary treatments for diabetes and other diseases (Eddouks, Chattopadhyay, De Feo, & Cho, 2014; Jamila & Mostafa, 2014; Mardani, Nasri, Rafieian-Kopaei, & Hajian, 2013). Although various plants have been traditionally used throughout history to reduce blood glucose and improve diabetes complications, there is not enough scientific information about some of them such as *Anacardium occidentale* L..

Anacardium occidentale Linn. (*A. occidentale* L.) or commonly known as cashew tree, is a popular tropical plant among Malaysian for its leaves, the cashew seed, and the cashew apple. Its young leaves are notably one of the commonly consumed vegetables by various ethnics such as Malays, Chinese, and Indian in Malaysia (Shukri et al., 2010).

As described earlier, the leaves of *A. occidentale* L. also possess an excellent source of bioactive compounds including polyphenols such as phenolic acids and flavonoids (Malviya, Jain, & Malviya, 2010; Nugroho, Malik, & Pramono, 2013; Ojezele & Agunbiade, 2013). Alkali and water extracts of *A. occidentale* L. contained predominantly gallic acid as well as protocatechuic, *p*-hydroxybenzoic, cinnamic, *p*-coumaric and ferulic acids (Kögel & Zech, 1985). Furthermore, extract of cashew shoots has been identified to have flavonol glycoside, with the highest constituent of kaempferol-3-O-glucoside, followed by kaempferol-3-*O*-arabinofuranoside and quercetin-3-*O*-glucoside (Shukri & Alan, 2010).

In the carbohydrate metabolism, polyphenols such as phenolic acids, flavonoids, and tannins play a role in inhibiting α-amylase, a glycoside hydrolase enzyme that if attenuated can slow down the breakdown of long-chain carbohydrates to glucose, this leads to a reduction in the rate at which glucose enters the blood stream , thus preventing a sudden rise in postprandial blood glucose levels (Jang & Moon, 2011; Laughlin et al., 1995; Nair, Kavrekar, & Mishra, 2013; Ranilla, Kwon, Apostolidis, & Shetty, 2010).

Polyphenols are also able to regulate postprandial glucose levels through the inhibition of Dipeptidyl peptidase IV (DPPIV), a serine protease that localizes on the cell surface of various tissues, including small intestine (Avila et al., 2017). By inhibiting DPPIV, it prevents the rapid degradation of incretins such as glucagon-like peptide 1 (GLP-1) and gastric inhibitory peptide (GIP) that play a vital role in blood glucose control. GLP-1 and GIP actions include stimulating insulin secretion, lowering glucagon concentration and slowing gastric emptying (Barnett, 2006; Nadkarni, Chepurny, & Holz, 2014).

For this reason, this study sought to determine whether *A. occidentale* leaves extracts to play a role in the inhibition of α-amylase and DPPIV enzymes. Here we compared the enzyme-inhibitory activities of the extracts between free, soluble esters, and insoluble bound phenolic fractions as well as between young and mature leaves. It is anticipated that these findings could provide some possible mechanisms by which they are used in the management and prevention of type 2 diabetes mellitus.

1.3 RESEARCH OBJECTIVES

The main objective of this study was to investigate the role of bioactive compounds from *A. occidentale* L.in inhibiting of α-amylase and dipeptidyl peptidase IV(DPPIV). In order to achieve the main objective, this project was subdivided into several specific objectives:

1. To extract free, soluble ester and insoluble bound of phenolic fractions of young and mature leaves of *A.occidentale* L.
2. To identify and quantify the polyphenols compounds (selected phenolic compounds) using HPLC-UV-Vis.
3. To determine bioactivities of leaves of *A. occidentale* L.on inhibition of α-amylase and DPPIV enzymes by maturity of leaves and phenolic fractions.

1.4 RESEARCH METHODOLOGY

The research was a laboratory-based experimental work use the young and mature leaves of *A. occidentale* L. to discover the potential to inhibit α- amylase and DPPIV. The flowchart of experimental studies is shown in Figure 1.1. The detailed methodology is described in chapter three of the thesis.

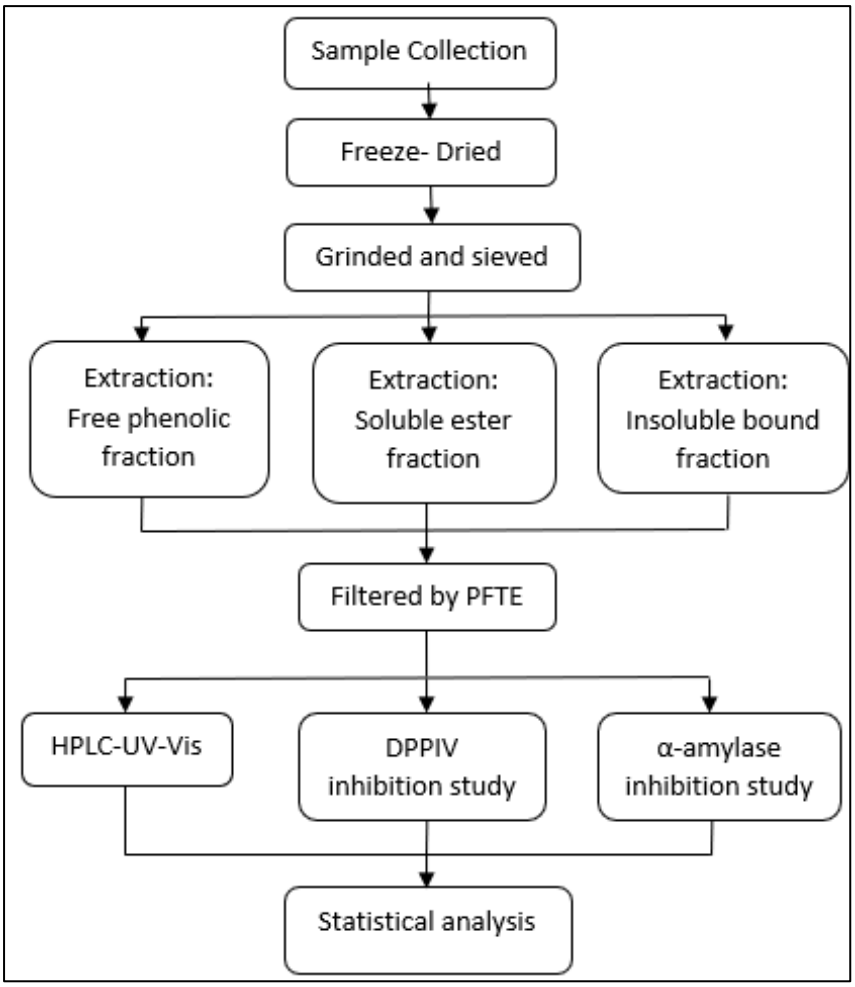

Figure 1.1 Flowchart of Experimental Studies.

1.5 DISSERTATION ORGANISATION

Chapter one is about an introduction to the research, provided with the background of the study, problem statements, and objectives. Chapter two is about the review of the literature concerning diabetes, therapeutic approach, enzymes, previous research on enzymes, medicinal plants, *A. occidentale* L., and polyphenols content in *A. occidentale* L.. Chapter three provides the materials and method used in the study. Chapter four presents the results and discusses the findings obtained from the research work with several comparisons from the previous research. Last but not least, chapter

five is about the conclusion by the objectives coupled with future recommendations on how to improve research.

CHAPTER TWO

LITERATURE REVIEWS

2.1 DIABETES MELLITUS

Diabetes mellitus (DM) cases are arising in the 21st century, and it is one of the major causes of death in humans after AIDS, cancer, and cerebrovascular diseases (Dey, Mitra, Katakam, & Singla, 2014). It has been projected that 642 million people aged 20- 79 years around the globe would suffer from diabetes by the year 2040, and diabetes will be one of the major causes of death globally (Ogurtsova et al., 2017).

Diabetes mellitus is a complex metabolic disorder, and patients diagnosed with it require continuous medical care and clinical appointments with treatment aiming towards multifactorial risk reduction plans beyond glycaemic control (Cameron, 2006). A lot of numbers of diabetes are producing and showing no symptom (Olokoba, Obateru, & Olokoba, 2012). However, common symptoms include increased thirst, polydipsia, increased frequency of urination, fatigue, loss of weight, blurred vision and increased risk of infection (Nolan, Damm, & Prentki, 2011).

2.1.1 Classification of Diabetes

Classification of diabetes is essential to step in selecting therapeutic management. The classical classification of diabetes mellitus is type 1 and type 2. However, some individuals are not able to be classified as suffering from type 1 or type 2 diabetes during the first clinical check-up. The traditional paradigms are type 2 diabetes exclusive in adulthood and type 1 diabetes exclusive in childhood are not entirely